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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>6</sup>:</b>  <b>C12N</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 97/38087</b>  <b>(43) International Publication Date:</b> 16 October 1997 (16.10.97)
<b>(21) International Application Number:</b> PCT/US97/06010  <b>(22) International Filing Date:</b> 4 April 1997 (04.04.97)  <b>(30) Priority Data:</b> 08/628,594                      5 April 1996 (05.04.96)                      US 08/668,953                      24 June 1996 (24.06.96)                      US 08/679,640                      12 July 1996 (12.07.96)                      US  <b>(71) Applicants (for all designated States except US):</b> CHIRON VIAGENE, INC. [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US). WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DUBENSKY, Thomas, W., Jr. [US/US]; P.O. Box 675205, Rancho Santa Fe, CA 92067 (US). POLO, John, M. [US/US]; 221 Witham Road, Encinitas, CA 92024 (US). BELLI, Barbara, A. [US/US]; 5850 Despejo Place, San Diego, CA 92124 (US). SCHLESINGER, Sondra [US/US]; 6320 McPherson Street, St. Louis, MO 63105 (US). DRYGA, Sergey A. [RU/US]; 6334 Southwood Avenue #3, St. Louis, MO 63105 (US). FROLOV, Ilya [RU/US]; 7515 Cromwell Drive #2SE, St. Louis, MO 63105 (US).		<b>(74) Agents:</b> McMASTERS, David, D. et al.; Seed and Berry L.L.P., 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> RECOMBINANT ALPHAVIRUS-BASED VECTORS WITH REDUCED INHIBITION OF CELLULAR MACROMOLECULAR SYNTHESIS  <b>(57) Abstract</b>  Isolated nucleic acid molecules are disclosed, comprising an alphavirus nonstructural protein gene which, when operably incorporated into a recombinant alphavirus particle, eukaryotic layered vector initiation system, or RNA vector replicon, has a reduced level of vector-specific RNA synthesis, as compared to wild-type, and the same or greater level of proteins encoded by RNA transcribed from the viral junction region promoter, as compared to a wild-type recombinant alphavirus particle. Also disclosed are RNA vector replicons, alphavirus vector constructs, and eukaryotic layered vector initiation systems which contain the above-identified nucleic acid molecules.		

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DescriptionRECOMBINANT ALPHAVIRUS-BASED VECTORS WITH REDUCED  
INHIBITION OF CELLULAR MACROMOLECULAR SYNTHESIS

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Statement of Government Interest

This invention has been made in part with government support under grant number AI 17377, awarded by the National Institutes of Health. The government may have certain rights in the invention.

10

Technical Field of the Invention

The present invention relates generally to recombinant DNA technology; and more specifically, to the development of recombinant vectors useful for directing the expression of one or more heterologous gene products.

15

Background of the Invention

Alphaviruses comprise a set of serologically related arthropod-borne viruses of the *Togaviridae* family. These viruses are distributed worldwide, and persist in nature through a mosquito to vertebrate cycle. Birds, rodents, horses, primates, and humans are among the defined alphavirus vertebrate reservoir/hosts.

Twenty-six known viruses and virus subtypes have been classified within the alphavirus genus utilizing the hemagglutination inhibition (HI) assay. This assay segregates the 26 alphaviruses into three major complexes: the Venezuelan equine encephalitis (VEE) complex, the Semliki Forest (SF) complex, and the western equine encephalitis (WEE) complex. In addition, four other viruses, eastern equine encephalitis (EEE), Barmah Forest, Middelburg, and Ndumu, receive individual classification based on the HI serological assay.

Members of the alphavirus genus also are classified based on their relative clinical features in humans: alphaviruses associated primarily with encephalitis, and alphaviruses associated primarily with fever, rash, and polyarthritis.

30

Included in the former group are the VEE and WEE complexes, and EEE. In general, infection with this group can result in permanent sequelae, including behavior changes and learning disabilities, or death. In the latter group is the SF complex, comprised of the individual alphaviruses Semliki Forest, Sindbis, Ross River, Chikungunya, O'nyong-nyong, and Mayaro. With respect to this group, although serious epidemics have been reported, infection is in general self-limiting, without permanent sequelae.

Sindbis virus is the prototype member of the *Alphavirus* genus of the *Togaviridae* family. Its replication strategy after infection of cells (see Figure 1) has been well characterized in chicken embryo fibroblasts (CEF) and baby hamster kidney (BHK) cells, where Sindbis virus grows rapidly and to high titer, and serves as a model for other alphaviruses. Briefly, the genome from Sindbis virus (like other alphaviruses) is an approximately 12 kb single-stranded positive-sense RNA molecule which is capped and polyadenylated, and contained within a virus-encoded capsid protein shell. The nucleocapsid is further surrounded by a host-derived lipid envelope into which two viral-specific glycoproteins, E1 and E2, are inserted and anchored to the nucleocapsid. Certain alphaviruses (e.g., SF) also maintain an additional protein, E3, which is a cleavage product of the E2 precursor protein, PE2. After virus particle absorption to target cells, penetration, and uncoating of the nucleocapsid to release viral genomic RNA into the cytoplasm, the replicative process is initiated by translation of the nonstructural proteins (nsPs) from the 5' two-thirds of the viral genome. The four nsPs (nsP1-nsP4) are translated directly from the genomic RNA template as one of two polyproteins (nsP123 or nsP1234), and processed post-translationally into monomeric units by an active protease in the C-terminal domain nsP2. A leaky opal (UGA) codon present between nsP3 and nsP4 of most alphaviruses accounts for a 10 to 20% abundance of the nsP1234 polyprotein, as compared to the nsP123 polyprotein. Both of the nonstructural polyproteins and their derived monomeric units may participate in the RNA replicative process, which involves binding to the conserved nucleotide sequence elements (CSEs) present at the 5' and 3' ends, and a junction region subgenomic promoter located internally in the genome (discussed further below).

The positive strand genomic RNA serves as template for the nsP-catalyzed synthesis of a full-length complementary negative strand. Synthesis of the complementary negative strand is catalyzed after binding of the nsP complex to the 3' terminal CSE of the positive strand genomic RNA. The negative strand, in turn, serves as template for the synthesis of additional positive strand genomic RNA and an abundantly expressed 26S subgenomic RNA, initiated internally at the junction region promoter. Synthesis of additional positive strand genomic RNA occurs after binding of the nsP complex to the 3' terminal CSE of the complementary negative strand genomic RNA template. Synthesis of the subgenomic mRNA from the negative strand genomic RNA template, is initiated from the junction region promoter. Thus, the 5' end and junction region CSEs of the positive strand genomic RNA are functional only after they are transcribed into the negative strand genomic RNA complement (*i.e.*, the 5' end CSE is functional when it is the 3' end of the genomic negative stranded complement). The structural proteins (sPs) are translated from the subgenomic 26S RNA, which represents the 3' one-third of the genome, and like the nsPs, are processed post-translationally into the individual proteins.

Several groups have suggested utilizing certain members of the alphavirus genus as an expression vector, including, for example, Sindbis virus (Xiong et al., *Science* 243:1188-1191, 1989; Hahn et al., *Proc. Natl. Acad. Sci. USA* 89:2679-2683, 1992; Dubenay et al., *J. Virol* 70:508-519, 1996), Semliki Forest virus (Liljestrom, *Bio/Technology* 9:1356-1361, 1991), and Venezuelan Equine Encephalitis virus (Davis et al., *J. Cell. Biochem. Suppl.* 19A:10, 1995). In addition, one group has suggested using alphavirus-derived vectors for the delivery of therapeutic genes *in vivo*. One difficulty, however, with the above-referenced vectors is that inhibition of host cell-directed macromolecular synthesis (*i.e.*, protein or RNA synthesis) begins within a few hours after infection and cytopathic effects (CPE) occur within 12 to 16 hours post infection (hpi). Inhibition and shutoff of host cell protein synthesis begins within 2 hpi in BHK cells infected with recombinant viral particles, in the presence or absence of structural protein expression, suggesting that the early events after virus infection (*e.g.*,

synthesis of nsPs and minus strand RNA) may directly influence the inhibition of host cell protein synthesis and subsequent development of CPE and cell death.

SIN-1 is a variant strain derived from wild-type Sindbis, and was isolated from a culture of BHK cells persistently infected with Sindbis virus over a period of one month (Weiss et al. *J. Virol.* 33: 463-474, 1980). A pure SIN-1 virus stock obtained by expansion from a single plaque does not kill the BHK cells which it infects. Importantly, virus yields ( $>10^3$  PFU/cell) are the same in BHK cells infected with wild-type Sindbis virus or the variant SIN-1 virus. Thus, the principle phenotype of SIN-1 in infected BHK cells is characterized by production of wild-type levels of infectious virus in the absence of virus-induced cell death.

The present invention provides recombinant vectors with selected desirable phenotypes for use in a variety of applications, including for example, gene therapy and recombinant protein production, and further provides other related advantages.

#### Summary of the Invention

Briefly stated, the present invention provides RNA vector replicons, alphavirus vector constructs, eukaryotic layered vector initiation systems and recombinant alphavirus particles which exhibit reduced, delayed, or no inhibition of cellular macromolecular synthesis (e.g., protein or RNA synthesis), thereby permitting the use of these vectors for protein expression, gene therapy and the like, with reduced, delayed, or no development of CPE or cell death. Such vectors may be constructed from a wide variety of alphaviruses (e.g., Semliki Forest virus, Ross River virus, Venezuelan equine encephalitis virus or Sindbis virus), and designed to express numerous heterologous sequences (e.g., a sequence corresponding to protein, a sequence corresponding to antisense RNA, a sequence corresponding to non-coding sense RNA, or a sequence corresponding to ribozyme).

Within one aspect of the invention, isolated nucleic acid molecules are provided comprising an altered alphavirus nonstructural protein gene which, when operably incorporated into a recombinant alphavirus, increases the time required to

reach 50% inhibition of host-cell directed macromolecular synthesis following expression in mammalian cells, as compared to a wild-type alphavirus. As utilized within the context of the present invention, "altered alphavirus nonstructural protein gene" refers to a gene which, when operably incorporated into an alphavirus RNA vector replicon, recombinant alphavirus particle, or eukaryotic layered vector initiation system, produces the desired phenotype (*e.g.*, reduced, delayed or no inhibition of cellular macromolecular synthesis). Such altered alphavirus nonstructural protein genes will have one or more nucleotide substitutions, deletions, or insertions, which alter the nucleotide sequence from that of the wild-type alphavirus gene. The gene may be derived either artificially (*e.g.*, from directed selection procedures; *see* Example 2 below), or from naturally occurring viral variants (*see* Example 1 below). In addition, it should be understood that when the isolated nucleic acid molecules of the present invention are incorporated into an alphavirus RNA vector replicon, recombinant alphavirus particle, or eukaryotic layered vector initiation system as discussed above, that they may, within certain embodiments, substantially increase the time required to reach 50% inhibition of host-cell directed macromolecular synthesis, up to and including substantially no detectable inhibition of host-cell directed macromolecular synthesis. Assays suitable for detecting percent inhibition of host-cell directed macromolecular synthesis include, for example, that described within Example 1.

Within other aspects of the invention, isolated nucleic acid molecules are provided comprising an altered alphavirus nonstructural protein gene which, when operably incorporated into a recombinant alphavirus particle, eukaryotic layered vector initiation system, or RNA vector replicon, results in a reduced level (*e.g.*, 2-fold, 5-fold, 10-fold, 50-fold or more than 100-fold) of vector-specific RNA synthesis as compared to the wild-type, and the same or greater level of protein encoded by RNA transcribed from the viral junction region promoter, as compared to a wild-type recombinant alphavirus particle, wild-type eukaryotic layered vector initiation system, or wild-type RNA vector replicon. Representative assays for quantitating RNA levels include [<sup>3</sup>H] uridine incorporation as described in Example 1, or RNA accumulation as detected by Northern Blot analysis (*see* Example 4). Representative assays for quantitating protein

levels include scanning densitometry (see Example 4) and various enzymatic assays (see Examples 3-5).

Within one embodiment of the above, the isolated nucleic acid molecule encodes nonstructural protein 2 (nsP2). Within a further embodiment, the isolated  
5 nucleic acid molecule has a mutation in the LXPGG motif of nsP2.

Within another aspect of the invention, expression vectors are provided comprising a promoter operably linked to one of the above-described nucleic acid molecules. Within one embodiment, the expression vector further comprises a polyadenylation sequence or transcription termination sequence 3' to the nucleic acid  
10 molecule.

Within yet another aspect of the present invention, alphavirus vector constructs are provided, comprising a 5' promoter which initiates synthesis of viral RNA *in vitro* from cDNA, a 5' sequence which initiates transcription of alphavirus RNA, a nucleic acid molecule which operably encodes all four alphaviral nonstructural  
15 proteins including an isolated nucleic acid molecule as described above, an alphavirus RNA polymerase recognition sequence and a 3' polyadenylate tract. Within one embodiment, such constructs further comprise a selected heterologous sequence downstream of and operably linked to a viral junction region.

Within yet other aspects of the present invention, RNA vector replicons  
20 capable of translation in a eukaryotic system are provided, comprising a 5' sequence which initiates transcription of alphavirus RNA, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, including the isolated nucleic acid molecules discussed above, an alphavirus RNA polymerase recognition sequence and a  
3' polyadenylate tract. Within another embodiment, such RNA vector replicons further  
25 comprise a selected heterologous sequence downstream of and operably linked to a viral junction region. Within further aspects of the invention, host cells are provided which contain one of the RNA vector replicons described herein. Within additional aspects of the invention, pharmaceutical compositions are provided comprising RNA vector replicons as described above and a pharmaceutically acceptable carrier or diluent.

Within other aspects of the invention, recombinant alphavirus particles are provided, comprising one or more alphavirus structural proteins, a lipid envelope, and an RNA vector replicon as described herein. Within one embodiment, one or more of the alphavirus structural proteins are derived from a different alphavirus than the  
5 alphavirus from which the RNA vector replicon was derived. Within other embodiments, the alphavirus structural protein and lipid envelopes are derived from different species. Within further aspects, pharmaceutical compositions are provided comprising a recombinant alphavirus particle as disclosed above and a pharmaceutically acceptable carrier or diluent. Further, mammalian cells infected with such recombinant  
10 alphavirus particles are also provided.

Within certain embodiments of the invention, the above described vectors or particles may further comprise a resistance marker which has been fused, in-frame, with the heterologous sequence. Representative examples of such resistance markers include hygromycin phosphotransferase and neomycin.

15 Within other aspects of the present invention, methods are provided for selecting alphavirus or recombinant alphavirus vector variants which exhibit the phenotype described herein of reduced, delayed, or, no inhibition of host cell directed macromolecular synthesis. Representative examples of such methods include the use of selectable drug or antigenic markers and are provided in more detail below in Example  
20 2.

Within other aspects of the present invention, Togavirus capsid particles are provided which contain substantially no genomic (*i.e.*, wild-type virus genome) or RNA vector replicon nucleic acids. Representative examples of Togaviruses include, for example alphaviruses and rubiviruses (*e.g.*, rubella). Within certain embodiments,  
25 the capsid particles further comprise a lipid envelope containing one or more alphavirus glycoproteins. Within other embodiments, the capsid particle further comprises an alphavirus envelope (*i.e.*, the lipid bilayer and the glycoprotein complement). Within related aspects of the present invention, pharmaceutical compositions are provided comprising the above noted capsid particles (with or without a lipid bilayer (*e.g.*, viral  
30 envelope containing alphavirus glycoproteins)) along with a pharmaceutically

acceptable carrier or diluent. Within further aspects, such capsid particles (with or without a lipid bilayer (e.g., viral envelope containing alphavirus glycoproteins)) or pharmaceutical compositions may be utilized as a vaccinating agent in order to induce an immune response against a desired togavirus.

5                   Within further aspects of the invention, inducible promoters are provided comprising a core RNA polymerase promoter sequence, an operably linked nucleic acid sequence that directs the DNA binding of a protein that activates transcription from the core promoter sequence, and an operably linked nucleic acid sequence that directs the DNA binding of a protein that represses transcription from the core promoter sequence.  
10   Such promoters may be utilized in the gene delivery vehicles described herein, as well as a wide variety of other vectors known to those skilled in the art.

                  Within other aspects, alphavirus structural protein expression cassettes are provided comprising a 5' promoter which initiates synthesis of viral RNA from DNA, a nucleic acid molecule which encodes one or more functional alphavirus  
15   structural proteins, a selectable marker operably linked to transcription of the expression cassette, and optionally, a 3' sequence which controls transcription termination. Within one embodiment, such expression cassettes further comprise a 5' sequence which initiates transcription of alphavirus RNA, a viral junction region promoter, and an alphavirus RNA polymerase recognition sequence. Within another embodiment, the  
20   expression cassette further comprises a catalytic ribozyme processing sequence, post-translational transcriptional regulatory elements which facilitate RNA export from the nucleus, and/or elements which permit translation of multicistronic mRNA, selected from the group consisting of Internal Ribosome Entry Site elements, elements promoting ribosomal read through and BiP sequence. Within other embodiments, the  
25   selectable marker is operably linked to a 5' promoter capable of initiating synthesis of alphavirus RNA from cDNA. Within further embodiments, the selectable marker is positioned downstream from a junction region promoter and from the nucleic acid molecule which encodes alphavirus structural proteins. Within yet other embodiments,  
30   embodiment, the alphavirus structural protein expression cassette further comprises an



alphavirus capsid protein gene or other sequence (e.g., a tobacco etch virus or "TEV" leader) which is capable of enhancing translation of one or more functional alphavirus structural protein genes located 3' to the enhancer sequence. Preferably, the capsid protein gene sequence is derived from a different alphavirus than that from which the  
5 sequence encoding the alphavirus structural genes is obtained.

Within yet other aspects of the invention, alphavirus packaging cell lines are provided comprising a cell containing an alphavirus structural protein expression cassette as described above. In certain embodiments, the alphavirus packaging cell lines are stably transformed with the alphavirus structural protein expression cassettes  
10 provided herein. Within related aspects, alphavirus producer cell lines are provided comprising a cell which contains a stably transformed alphavirus structural protein expression cassette, and a vector selected from the group consisting of RNA vector replicons, alphavirus vector constructs and eukaryotic layered vector initiation systems.

Within yet other aspects of the present invention, eukaryotic layered  
15 vector initiation systems are provided, comprising a 5' promoter capable of initiating *in vivo* the 5' synthesis of RNA from cDNA, a sequence which initiates transcription of alphavirus RNA following the 5' promoter; a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, including an isolated nucleic acid molecule as discussed above, an alphavirus RNA polymerase recognition sequence, and  
20 a 3' polyadenylate tract. Representative examples of suitable 5' promoters include RNA polymerase I promoters, RNA polymerase II promoters, RNA polymerase III promoters, the HSV-TK promoter, RSV promoter, tetracycline inducible promoter, MoMLV promoter, a SV40 promoter and a CMV promoter. Within preferred embodiments, the 5' promoter is an inducible promoter as described herein.

25 Within certain embodiments, eukaryotic layered vector initiation systems are provided which further comprise a heterologous sequence operably linked to a viral junction region, and/or a post-transcriptional regulatory element which facilitates RNA export from the nucleus. Within further embodiments, the eukaryotic layered vector initiation systems provided herein may further comprise a transcription termination  
30 signal.

Within related aspects, the present invention also provides host cells (e.g., vertebrate or insect) containing a stably transformed eukaryotic layered vector initiation system as described above. Within further aspects of the present invention, methods for delivering a selected heterologous sequence to a vertebrate or insect are provided, comprising the step of administering to a vertebrate or insect an alphavirus vector construct, RNA vector replicon, recombinant alphavirus particle, or a eukaryotic layered vector initiation system as described herein. Within certain embodiments, the alphavirus vector construct, RNA vector replicon, recombinant alphavirus particle or eukaryotic layered vector initiation system is administered to cells of the vertebrate *ex vivo*, followed by administration of the vector or particle-containing cells to a warm-blooded animal.

Within other aspects, pharmaceutical compositions are provided comprising a eukaryotic layered vector initiation system as discussed above, and a pharmaceutically acceptable carrier or diluent. Within certain embodiments, the pharmaceutical composition is provided as a liposomal formulation.

Within further aspects, methods of making recombinant alphavirus particles are provided, comprising the steps of (a) introducing a vector such as a eukaryotic layered vector initiation system, RNA vector replicon, or alphavirus vector particle as described above into a population of packaging cells under conditions and for a time sufficient to permit production of recombinant alphavirus particles, and (b) harvesting recombinant alphavirus particles. Within related aspects, methods of making a selected protein are provided, comprising the steps of (a) introducing a vector which encodes a selected heterologous protein, such as a eukaryotic layered vector initiation system, RNA vector replicon or alphavirus vector particle described above, into a population of packaging cells, or other cells under conditions and for a time sufficient to permit production of the selected protein, and (b) harvesting protein produced by the vector containing cells. Within yet other aspects, methods of making a selected protein are provided, comprising the step of introducing a eukaryotic layered vector initiation system which is capable of producing a selected heterologous protein into a host cell, under conditions and for a time sufficient to permit expression of the

selected protein. Within further aspects, host cell lines are provided which contain a RNA vector replicon as described herein.

Within yet other aspects of the present invention, alphavirus vaccines are provided, comprising one of the above-described alphavirus vector constructs, RNA  
5 vector replicons, eukaryotic vector initiation systems, or recombinant alphavirus particles, which may or may not express one of the heterologous sequences provided herein (*e.g.*, they may be utilized solely as a vaccine for treating or preventing alphaviral diseases). For example, within one embodiment of the invention, recombinant togavirus particles are provided which have substantially no nucleic acid  
10 or RNA vector replicon nucleic acid. Within a further embodiment, recombinant togavirus particles are provided which contain heterologous viral nucleic acids (*i.e.*, from a different virus than the togavirus particle). Within yet another embodiment, the recombinant togavirus particle is T=3 or greater.

Within further aspects of the invention, recombinant chimeric togavirus  
15 particles (either empty, or containing nucleic acids) are provided wherein the viral particle has viral structural components obtained or derived from different Togaviridae (*e.g.*, the capsid protein and glycoprotein is obtained from different alphavirus sources).

These and other aspects and embodiments of the invention will become evident upon reference to the following detailed description and attached figures. In  
20 addition, various references are set forth herein that describe in more detail certain procedures or compositions (*e.g.*, plasmids, sequences, etc.), and are therefore incorporated by reference in their entirety as if each were individually noted for incorporation.

## 25 Brief Description of the Figures

Figure 1 is a schematic illustration of Sindbis genome organization and replication strategy.

Figure 2 is a graph of virus release from BHK cells infected at an MOI of 10 with SIN-1, SIN-1/nsP1-4, Toto1101, or Sin-1/nsP2 viruses. Culture supernates

were collected at 3, 6, 9 and 12 hours post-infection. Virus titers were determined by plaque assay.

Figure 3 is a graph depicting viral RNA synthesis in BHK cells following infection by Toto1101, SIN-1/nsP2, SIN-1/nsP1-4, or SIN-1 virus. Cells  
5 were infected at an MOI 10 and at 1 hour post-infection, actinomycin D and <sup>3</sup>H-uridine were added. At 3, 6, 9, and 12 hpi the amount of <sup>3</sup>H-uridine incorporation was determined.

Figure 4 is a graph depicting viral RNA synthesis in BHK cells infected by SIN-1/nsP1, SIN-1/nsP2, SIN-1/nsP3, SIN-1/nsP3-4, SIN-1/nsP4, SIN-1/nsP2-C,  
10 SIN-1/nsP2-N, Totol101, SIN-1, or SIN-1/nsP1-4. The levels of <sup>3</sup>H-uridine incorporation are expressed relative to wild-type (Toto 1101) infection.

Figure 5 is a graph depicting the shut-off of host cell protein synthesis in BHK cells infected by SIN-1nsP1-4, SIN-1, SIN-1nsP2, or Totol101 viruses.

Figure 6 is the cDNA sequence of 8000 bases of SIN-1 virus (SEQ. ID  
15 NO. 101).

Figure 7 is the cDNA sequence of 8000 bases of SINCG virus (SEQ. ID NO. 102).

Figure 8 is the cDNA sequence of Toto 1101 virus (SEQ. ID NO. 103).

Figure 9A is a northern blot of RNAs isolated from BHK-21 cells that  
20 were transfected with pBG/SIN-1 ELVS 1.5-SEAP or pBG/wt ELVS 1.5-SEAP plasmid DNAs, and hybridized with a radiolabeled viral RNA probe. Figure 9B is a graph depicting a 7 day timecourse of alkaline phosphatase expression in BHK cells transfected with pBG/SIN-1 ELVS 1.5-SEAP or pBG/wt ELVS 1.5-SEAP plasmid DNAs.

25 Figure 10 is a graph depicting a 4 day timecourse of luciferase expression in BHK cells transfected with pBG/SIN-1 ELVS 1.5-luc or pBG/wt ELVS 1.5-luc plasmid DNAs.

Figure 11A is a northern blot of RNAs isolated from BHK-21 cells that were transfected with pBG/SIN-1 ELVS-1.5-β-gal or pBG/wt ELVS 1.5-β-gal plasmid  
30 DNAs, and hybridized with a radiolabeled viral RNA probe. Figure 11B is a western

blot detecting  $\beta$ -gal expression in BHK-21 cells transfected with either pBG/SIN-1 ELVS-1.5- $\beta$ -gal or pBG/wt ELVS 1.5- $\beta$ -gal plasmid DNAs. Figure 11C is a graph depicting a 5 day timecourse of alkaline phosphatase expression in BHK cells transfected with pBG/SIN-1 ELVS-1.5- $\beta$ -gal or pBG/wt ELVS 1.5- $\beta$ -gal plasmid DNAs.

Figure 12A & B are graphs depicting  $\beta$ -gal expression in HT1080 and BHK-21 cells transfected with ELVS  $\beta$ -gal vectors with or without HBV PRE sequences, as measured by RLU (relative light units).

Figure 13 is a schematic illustration of RNA amplification, structural protein expression, and vector packaging by vector inducible alphavirus packaging cell lines.

Figure 14 is a schematic illustration of vector inducible structural protein expression cassettes used in the generation of alphavirus packaging cell lines.

Figure 15 is a graph depicting luciferase vector packaging (transfer of expression) by different alphavirus packaging cell lines.

Figure 16 is a western blot depicting vector inducible structural protein expression by an alphavirus packaging cell line.

Figure 17A is a graph depicting luciferase vector packaging by C6/36 mosquito cells containing the pDCMV-intSINrbz structural protein expression cassette. Figure 17B is a graph depicting luciferase vector packaging by human 293 packaging cells stably transformed with plasmid pBGSVCMVdlneo.

Figure 18 is a graph depicting luciferase vector packaging by different alphavirus packaging cell lines.

Figure 19 is an RNA gel autoradiograph depicting  $^3\text{H}$  uridine-labeled RNAs from BHK cells infected with SINrep/LacZ vector particles produced from an alphavirus packaging cell line.

Figure 20 is a protein gel autoradiograph depicting  $^{35}\text{S}$  methionine-labeled proteins from BHK cells infected with SINrep/LacZ vector particles produced from an alphavirus packaging cell line.

Figure 21 is a western blot depicting vector inducible capsid protein expression following  $\beta$ -gal or glycoprotein vector transfection of a capsid cell line.

Figure 22 is a schematic illustration of the region of structural protein expression cassettes comprising a wild-type or deletion mutant Ross River virus capsid protein gene.

Figure 23 is a schematic illustration of vector inducible structural protein expression cassettes containing a wild-type or deletion mutant Ross River virus capsid protein gene.

Figure 24 is a schematic illustration of vector packaging by "split" structural protein gene expression cassettes which contain a Ross River virus capsid protein gene sequence upstream of the Sindbis virus glycoprotein genes.

Figure 25 is a schematic illustration of vector packaging by "split" structural protein gene expression cassettes which contain a Ross River virus capsid protein gene sequence upstream of the Sindbis virus glycoprotein genes on one cassette, and the Sindbis virus capsid protein gene in a separate cassette.

Figure 26 is a table showing the results of vector particle packaging using the above "split" structural protein gene expression cassettes.

Figure 27 is a schematic illustration of the use of alphavirus packaging cell lines for the amplification of packaged vector particle preparations and the large scale production of recombinant protein.

Figure 28 is a graph depicting the amplification and production of  $\beta$ -gal protein over time using alphavirus packaging cell lines.

Figure 29 is a schematic illustration of the use of a tetracycline regulated promoter system to control expression of alphavirus vector RNA from cDNA *in vivo*.

Figure 30 is a schematic illustration of the use of a linked transcriptional repressor and a transcriptional inducer/activator regulated promoter system to control expression of alphavirus vector RNA from cDNA *in vivo*.

Figure 31A & B are autoradiographs of [ $^3$ H]uridine-labeled RNAs electrophoresed on denaturing glyoxal gels that were isolated from BHK cell electroporated with SINrep/LacZ replicon and DH RNAs from various RRV capsid

containing DH constructs, and from the vector particles present in the culture fluids at 18 hours post electroporation.

Figure 32A & B are protein gel autoradiographs depicting <sup>35</sup>S methionine-labelled proteins from BHK cells electroporated with SINrep/LacZ replicon and DH RNAs from various RRV capsid containing DH constructs, and from the vector particles present in the culture fluids at 18 hours post electroporation.

Figure 33A-D are Kyte-Doolittle hydrophobicity plots of various Ross River virus (RRV) capsid proteins, expressed from the wild-type gene (A) and three deletion mutants CΔ1rrv, CΔ2rrv, and CΔ3rrv (B-D, respectively).

Figure 34 is a schematic that illustrates the amino-terminus RRV capsid proteins expressed from the wild-type gene (SEQ. ID NO. 114), and three deletion mutants CΔ1rrv (SEQ. ID NO. 115), CΔ2rrv (SEQ. ID NO. 116), and CΔ3rrv (SEQ. ID NO. 117). The lysine residues deleted in the RRV capsid gene mutants are indicated.

Figure 35 is a graph that illustrates the relative levels of [<sup>35</sup>S]methionine and [<sup>3</sup>H]uridine incorporated into virus particles in BHK cells infected at high MOI with Toto1101 wild-type virus.

Figure 36 is a graph that illustrates the relative levels of [<sup>35</sup>S] methionine and [<sup>3</sup>H]uridine incorporated into virus particles in BHK cells electroporated with SINrep/lacZ and DH-BB (5' tRNA) Crrv DH RNAs.

Figure 37 is a graph that illustrates the relative levels of [<sup>35</sup>S]methionine and [<sup>3</sup>H]uridine incorporated into virus particles in BHK cells electroporated with SINrep/lacZ and RRV capsid deletion mutant DH-BB (5' tRNA) CΔ3rrv DH RNAs.

Figure 38 is a graph which compiles the results shown in Figures 35-37, depicting the relative levels of [<sup>35</sup>S]methionine and [<sup>3</sup>H]uridine incorporated into virus particles in BHK cells electroporated with SINrep/lacZ and DH RNAs, or infected with Toto1101 wild-type virus.

Figure 39 is a graph which illustrates luciferase vector packaging by BHK cells stably transformed with pBGSVCMVdlhyg.

### Definition of Terms

The following terms are used throughout the specification. Unless otherwise indicated, these terms are defined as follows:

"Genomic RNA" refers to RNA which contains all of the genetic information required to direct its own amplification or self-replication *in vivo*, within a target cell. To direct its own replication, the RNA molecule may: 1) encode one or more polymerase, replicase, or other proteins which may interact with viral or host cell-derived proteins, nucleic acids or ribonucleoproteins to catalyze the RNA amplification process; and 2) contain *cis* RNA sequences required for replication, which may be bound during the process of replication by its self-encoded proteins, or non-self-encoded cell-derived proteins, nucleic acids or ribonucleoproteins, or complexes between any of these components. An alphavirus-derived genomic RNA molecule should contain the following ordered elements: 5' viral or defective-interfering RNA sequence(s) required in *cis* for replication, sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), 3' viral sequences required in *cis* for replication, and a polyadenylate tract. The alphavirus-derived genomic RNA vector replicon also may contain a viral subgenomic "junction region" promoter which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of the subgenomic fragment, and sequences which, when expressed, code for biologically active alphavirus structural proteins (*e.g.*, C, E3, E2, 6K, E1). Generally, the term genomic RNA refers to a molecule of positive polarity, or "message" sense, and the genomic RNA may be of length different from that of any known, naturally-occurring alphavirus. In preferred embodiments, the genomic RNA does not contain the sequences which encode any alphaviral structural protein(s); rather those sequences are substituted with heterologous sequences. In those instances where the genomic RNA is to be packaged into a recombinant alphavirus particle, it must contain one or more sequences which serve to initiate interactions with alphavirus structural proteins that lead to particle formation, and preferably is of a length which is packaged efficiently by the packaging system being employed.



"Subgenomic RNA", or "26S" RNA, refers to a RNA molecule of a length or size which is smaller than the genomic RNA from which it was derived. The subgenomic RNA should be transcribed from an internal promoter whose sequences reside within the genomic RNA or its complement. Transcription of the subgenomic RNA may be mediated by viral-encoded polymerase(s), host cell-encoded polymerase(s), transcription factor(s), ribonucleoprotein(s), or a combination thereof. In preferred embodiments, the subgenomic RNA is produced from a vector according to the invention, and encodes or expresses the gene(s) or sequence(s) of interest. The subgenomic RNA need not necessarily have a sedimentation coefficient of 26.

10        "Alphavirus vector construct" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. Such vector constructs are comprised of a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also referred to as 5' CSE, in background), as well as sequences which, when expressed, code for biologically active alphavirus nonstructural proteins  
15 (e.g., nsP1, nsP2, nsP3, nsP4), and an alphavirus RNA polymerase recognition sequence (also referred to as 3' CSE, in background). In addition, the vector construct should include a viral subgenomic "junction region" promoter which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of the subgenomic fragment, and also a polyadenylate tract. The vector also may include  
20 sequences from one or more structural protein genes or portions thereof, extraneous nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, a 5' promoter which is capable of initiating the synthesis of viral RNA *in vitro* from cDNA, a heterologous sequence to be expressed, as well as one or more restriction sites for insertion of heterologous sequences.

25        "Alphavirus RNA vector replicon", "RNA vector replicon" and "replicon" refers to a RNA molecule which is capable of directing its own amplification or self-replication *in vivo*, within a target cell. To direct its own amplification, the RNA molecule may: 1) encode one or more polymerase, replicase, or other proteins which may interact with viral or host cell-derived proteins, nucleic acids or ribonucleoproteins  
30 to catalyze RNA amplification; and 2) contain *cis* RNA sequences required for

replication which may be bound by its self-encoded proteins, or non-self-encoded cell-derived proteins, nucleic acids or ribonucleoproteins, or complexes between any of these components. In certain embodiments, the amplification also may occur *in vitro*. An alphavirus-derived RNA vector replicon molecule should contain the following  
5 ordered elements: 5' viral sequences required in *cis* for replication (also referred to as 5' CSE, in background), sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), 3' viral sequences required in *cis* for replication (also referred to as 3' CSE, in background), and a polyadenylate tract. The alphavirus-derived RNA vector replicon also may contain a  
10 viral subgenomic "junction region" promoter which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of the subgenomic fragment, sequences from one or more structural protein genes or portions thereof, extraneous nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, as well as heterologous sequence(s) to be expressed. The source of RNA  
15 vector replicons in a cell may be from infection with a virus or recombinant alphavirus particle, or transfection of plasmid DNA or *in vitro* transcribed RNA.

"Recombinant Alphavirus Particle" refers to a virion unit containing an alphavirus RNA vector replicon. Generally, the recombinant alphavirus particle comprises one or more alphavirus structural proteins, a lipid envelope and an RNA  
20 vector replicon. Preferably, the recombinant alphavirus particle contains a nucleocapsid structure that is contained within a host cell-derived lipid bilayer, such as a plasma membrane, in which alphaviral-encoded envelope glycoproteins are embedded. The particle may also contain other components (*e.g.*, targeting elements such as biotin, other viral structural proteins, or other receptor binding ligands) which direct the  
25 tropism of the particle from which the alphavirus was derived, or other RNA molecules.

"Structural protein expression cassette" refers to a nucleic acid molecule which is capable of directing the synthesis of one or more alphavirus structural proteins. The expression cassette should include a 5' promoter which is capable of initiating  
*in vivo* the synthesis of RNA from cDNA, as well as sequences which, when expressed,  
30 code for one or more biologically active alphavirus structural proteins (*e.g.*, C, E3, E2.

6K, E1), and a 3' sequence which controls transcription termination. The expression cassette also may include a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also referred to as 5' CSE, in background), a viral subgenomic "junction region" promoter, and an alphavirus RNA polymerase recognition sequence (also referred to as 3' CSE, in background). In certain embodiments, the expression cassette also may include splice recognition sequences, a catalytic ribozyme processing sequence, a sequence encoding a selectable marker, a nuclear export signal, as well as a polyadenylation sequence. In addition, expression of the alphavirus structural protein(s) may, in certain embodiments, be regulated by the use of an inducible promoter.

10                "Stable Transformation" refers to the introduction of a nucleic acid molecule into a living cell, and long-term or permanent maintenance of that nucleic acid molecule in progeny cells through successive cycles of cell division. The nucleic acid molecule may be maintained in any cellular compartment, including, but not limited to, the nucleus, mitochondria, or cytoplasm. In preferred embodiments, the nucleic acid molecule is maintained in the nucleus. Maintenance may be intrachromosomal (integrated) or extrachromosomal, as an episomal event.

15                "Alphavirus packaging cell line" refers to a cell which contains an alphavirus structural protein expression cassette and which produces recombinant alphavirus particles after introduction of an alphavirus vector construct, RNA vector replicon, eukaryotic layered vector initiation system, or recombinant alphavirus particle. The parental cell may be of mammalian or non-mammalian origin. Within preferred embodiments, the packaging cell line is stably transformed with the structural protein expression cassette.

20                "Alphavirus producer cell line" refers to a cell line which is capable of producing recombinant alphavirus particles, comprising an alphavirus packaging cell line which also contains an alphavirus vector construct, RNA vector replicon, eukaryotic layered vector initiation system, or recombinant alphavirus particle. Preferably, the alphavirus vector construct is eukaryotic latered vector initiation system, and the producer cell line is stably transformed with the vector construct. In preferred  
25                30                embodiments, transcription of the alphavirus vector construct and subsequent

production of recombinant alphavirus particles occurs only in response to one or more factors, or the differentiation state of the alphavirus producer cell line.

"Defective helper construct" refers to an assembly which is capable of RNA amplification or replication, and expression of one or more alphavirus structural proteins in response to biologically active alphavirus nonstructural proteins supplied in *trans*. The defective helper construct should contain the following ordered elements: 5' viral or defective-interfering RNA sequences required in *cis* for replication, a viral subgenomic junction region promoter, sequences which, when expressed, code for one or more biologically active alphavirus structural proteins (e.g., C, E3, E2, 6K, E1), 3' viral sequences required in *cis* for replication, and a polyadenylate tract. The defective helper construct also may contain a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, a 3' sequence which controls transcription termination, splice recognition sequences, a catalytic ribozyme processing sequence, a sequence encoding a selectable marker, and a nuclear export signal.

15       "Eukaryotic Layered Vector Initiation System" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. The eukaryotic layered vector initiation system should contain a 5' promoter which is capable of initiating *in vivo* (i.e. within a cell) the synthesis of RNA from cDNA, and a nucleic acid vector sequence which is capable of directing its own replication in a eukaryotic cell and also expressing a heterologous sequence. The nucleic acid sequence which is capable of directing its own amplification may be of viral or non-viral origin. In certain embodiments, the nucleic acid vector sequence is an alphavirus-derived sequence and is comprised of a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also referred to as 5' CSE, in background), as well as sequences 25 which, when expressed, code for biologically active alphavirus nonstructural proteins (e.g., nsP1, nsP2, nsP3, nsP4), and an alphavirus RNA polymerase recognition sequence (also referred to as 3' CSE, in background). In addition, the vector sequence may include a viral subgenomic "junction region" promoter which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of 30 the subgenomic fragment, sequences from one or more structural protein genes or

portions thereof, extraneous nucleic acid molecule(s) which are of a size sufficient to allow optimal amplification, a heterologous sequence to be expressed, one or more restriction sites for insertion of heterologous sequences, as well as a polyadenylation sequence. The eukaryotic layered vector initiation system may also contain splice  
5 recognition sequences, a catalytic ribozyme processing sequence, a nuclear export signal, and a transcription termination sequence. In certain embodiments, *in vivo* synthesis of the vector nucleic acid sequence from cDNA may be regulated by the use of an inducible promoter.

"Alphavirus cDNA vector construct" refers to an assembly which is  
10 capable of directing the expression of a sequence(s) or gene(s) of interest. The vector construct is comprised of a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also referred to as 5' CSE), as well as sequences which, when expressed, code for biologically active alphavirus nonstructural proteins (*e.g.*, nsP1, nsP2, nsP3, nsP4), and an alphavirus RNA polymerase recognition sequence (also  
15 referred to as 3' CSE, in background). In addition, the vector construct should include a 5' promoter which is capable of initiating *in vivo* the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination. Within certain embodiments, the vector construct may further comprise a viral subgenomic "junction region" promoter which may, in certain embodiments, be modified in order to prevent,  
20 increase, or reduce viral transcription of the subgenomic fragment. The vector also may include sequences from one or more structural protein genes or portions thereof, extraneous nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, a heterologous sequence to be expressed, one or more restriction sites for insertion of heterologous sequences, splice recognition sequences, a catalytic ribozyme  
25 processing sequence, a nuclear export signal, as well as a polyadenylation sequence. In certain embodiments, *in vivo* synthesis of viral RNA from cDNA may be regulated by the use of an inducible promoter.

"Gene delivery vehicle" refers to a construct which can be utilized to deliver a gene or sequence of interest. Representative examples include alphavirus

RNA vector replicons, alphavirus vector constructs, eukaryotic layered vector initiation systems and recombinant alphavirus particles.

Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description which  
5 provides illumination of the practice of the invention.

#### Detailed Description of the Invention

As noted above, the present invention provides novel gene delivery vehicles including for example, RNA vector replicons, alphavirus vector constructs,  
10 eukaryotic layered vector initiation systems and recombinant alphavirus particles. Briefly, introduction of plasmid DNA-, *in vitro* transcribed RNA-, or particle-based vectors of the present invention into a cell, results in levels of heterologous gene expression that are equivalent, or higher, as compared to expression levels of wild-type derived alphaviral vectors. Unexpectedly however, the level of vector-specific RNA  
15 synthesized is at least about 5 to 10-fold lower in cultured cells which contain a gene delivery vehicle of the present invention, as compared to wild-type derived vectors. Furthermore, such gene delivery vehicles exhibit reduced, delayed, or no inhibition of host cell-directed macromolecular synthesis following introduction into a host cell, as compared to wild-type derived vectors.

20 As discussed in more detail below, the present invention provides:  
(A) sources of wild-type alphaviruses suitable for constructing the gene delivery vehicles of the present invention; (B) methods for selecting alphaviruses with a desired phenotype; (C) construction of alphavirus vector constructs and alphavirus RNA vector replicons; (D) construction of Eukaryotic Layered Vector Initiation Systems;  
25 (E) construction of recombinant alphavirus particles; (F) heterologous sequences which may be expressed by the gene delivery vehicles of the present invention; (G) construction of alphavirus packaging or producer cell lines; (H) pharmaceutical compositions; and (I) methods for utilizing alphavirus-based vectors.

A. Sources of Wild-Type Alphavirus

As noted above, the present invention provides a wide variety of alphavirus-based vectors (e.g., RNA vector replicons, alphavirus vector constructs, eukaryotic layered vector initiation systems and recombinant alphavirus particles), as well as methods for utilizing such vector constructs and particles. Briefly, sequences encoding wild-type alphaviruses suitable for use in preparing the above-described vectors can be readily obtained given the disclosure provided herein from naturally-occurring sources, or from depositories (e.g., the American Type Culture Collection, Rockville, Maryland). In addition, wild-type alphaviruses may be utilized for comparing the level of host-cell directed macromolecular synthesis in cells infected with the wild-type alphavirus, with the level of host-cell directed macromolecular synthesis in cells containing the gene delivery vehicles of the present invention.

Representative examples of suitable alphaviruses include Aura virus (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou virus (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan virus (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach virus (ATCC VR-927), Mayaro virus (ATCC VR-66, ATCC VR-1277), Middleburg virus (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu virus (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest virus (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248; *see also* CMCC #4640, described below), Tonate virus (ATCC VR-925), Trinita virus (ATCC VR-469), Una virus (ATCC VR-374), Venezuelan equine encephalomyelitis virus (ATCC VR-69, ATCC VR-923, ATCC VR-1250, ATCC VR-1249, ATCC VR-532), Western equine encephalomyelitis virus (ATCC VR-70, ATCC VR-1251, ATCC VR-622, ATCC VR-1252), Whataroa virus (ATCC VR-926), and Y-62-33 virus (ATCC VR-375).

For purposes of comparing levels of cellular macromolecular synthesis, the following plasmids may also be utilized as a standard source of wild-type alphavirus stocks. These plasmids include: for Semliki Forest Virus, pSP6-SFV4 (Liljestrom et al., *J. Virol.* 65:4107-4113, 1991); for Venezuelan equine encephalitis virus, pV2000

(Davis et al., *Vir.* 183:20-31, 1991); for Ross River virus, pRR64 (Kuhn et al., *Vir.* 182:430-441, 1991). Briefly, for these plasmids, virus can be obtained from BHK cells transfected with *in vitro* transcribed genomic RNA from the plasmids. For Sindbis virus, infectious virus may be isolated directly from BHK cells transfected with  
5 pVGELVIS (ATCC No. 75891) plasmid DNA, or alternatively, obtained as a wild-type virus stock (see deposit information provided below regarding ATCC No. VR-2526).

B. Selection of Alphaviruses With a Desired Phenotype

The duration of *in vivo* heterologous gene expression from alphavirus-  
10 based vectors is affected by several mechanisms, including inhibition of host cell-directed macromolecular synthesis. However, prior to the present invention, there had been no obvious method to select for or identify coding or non-coding vector viral-specific sequence changes that result in a non-cytopathic phenotype. Therefore, within one aspect of the present invention methods are provided for isolating and/or  
15 constructing alphavirus-derived gene delivery vehicles with reduced or no inhibition of host cell directed macromolecular synthesis.

1. Biological Selection of Virus Variants

a. Selection from Virus Stocks Containing DI Particles

20 One approach for isolating non-cytopathic alphavirus variants exploits the presence of defective interfering (DI) particles in wild-type virus preparations. Briefly, although certain RNA viruses, for example rhabdoviruses (*e.g.*, vesicular stomatitis virus) and alphaviruses (*e.g.*, Sindbis virus and Semliki Forest virus), are highly cytopathic, they can nevertheless establish long-term persistent infection in  
25 cultured cells in the presence of DI particles. DI particles, by definition, are derived from wild-type virus and contain one or more mutations (*e.g.*, deletions, rearrangements, nucleotide substitutions, *etc.*) from the wild-type genome which prevent autonomous replication by the DI. In general, the genome of DI particles is smaller and of a lower complexity compared to wild-type virus, and is deleted of  
30 protein-encoding regions while maintaining regions required *in cis* for replication. Such *cis* sequences often are duplicated and/or rearranged. In the case of certain alphaviruses



(*e.g.*, Sindbis virus), the sequence and organization of DI RNA genomes have been analyzed and found to contain a minimum of 50 nt from the extreme 3'-end of the wild-type virus genome, and at their 5'-ends, either a wild-type sequence or a cellular tRNA (*e.g.*, tRNA<sup>Asp</sup>) sequence, in addition to the viral sequence. In all cases, the propagation and maintenance of the mutated DI genomes requires the co-existence of parental helper virus in the infected cell. However, as a result of their genetic structure, DI genome replication is vastly superior and comparatively abundant to its wild-type counterpart. This characteristic results in interference of wild-type genome replication, the absence or low level production of infectious virus, and the establishment of long-term persistent infection of cells.

Therefore, as described below in Examples 1 and 2, the ability to establish long-term persistent infection in permissive cells (*e.g.*, mammalian cells, including cells of human origin) by infecting with a mixed alphavirus stock containing a population of DI particles provides a mechanism to isolate, over time, fully intact virus variants that are able to establish persistent infection, even in the absence of DI particles. Such infectious virus variants can be isolated from long-term persistently infected cultures by multiple rounds of plaque purification and have been found to initiate productive, persistent, and non-cytopathic infection in the host cells. Furthermore, the level of variant virus produced from such a productive, persistent, and non-cytopathic infection is indistinguishable from wild-type virus infection. This observation is in distinct contrast to the previous requirement for establishment of persistent infections with virus stocks containing a mixture of DI particles.

b. Selection from Virus Stocks Not Containing DI Particles

In addition to selection from virus stocks that contain defective-interfering particles, virus variants suitable for use within the present invention may be obtained from purified virus stocks (without DI particles) which are either subjected to random mutagenesis prior to infection of susceptible cultured cells or allowed to generate non-specific mutations during RNA replication with the cultured cells. Briefly, the initial virus stock may be obtained as a natural isolate or biological variant derived therefrom, or may be generated by transfecting cultured cells with an infectious

nucleic acid molecule comprising a genomic cDNA clone or *in vitro* transcribed RNA. If desired, the virus stock may then subjected to physical or chemical mutagenesis (although preferred, such mutagenesis is not required). In the case of chemical mutagenesis, preferred embodiments utilize a readily available mutagenic agent, for example nitrous acid, 5-azacytidine, N-methyl-N'-nitro-N-nitrosoguanidine, or ethylmethane sulfonate (Sigma, St. Louis, MO), prior to virus infection. Following random mutagenesis, specific selection procedures are applied to isolate virus variants possessing the desired phenotype, as described in more detail below in Example 2.

## 10           2.       Genetic Selection of Virus Variants

In a related approach, mutations may be obtained not using a virus stock, but rather, using cloned genomic cDNA of the virus that can be used subsequently to transcribe infectious viral RNA *in vitro* (for example, Sindbis virus (Rice et al., *J. Virol.* 61:3809-3819, 1987; Dubensky et al., *J. Virol.* 70:508-519, 1996, SFV (Liljeström et al., *J. Virol.* 65:4107-4113, 1991, VEE (Davis et al., *Virology* 183:20-31, 1991), Ross River virus (Kuhn et al., *Virology* 182:430-441, 1991), poliovirus (Van Der Werf et al., *Proc. Natl. Acad. Sci. USA* 83:2330-2334, 1986)) or *in vivo* (Sindbis virus (Dubensky et al., *ibid.*), poliovirus (Racaniello and Baltimore, *Science* 214:916-919, 1981)). Briefly, the infectious nucleic acid is introduced into susceptible cultured cells (e.g., mammalian cells, including cells of human origin) either directly or following mutagenesis performed using one of the above-referenced methods. Alternatively, vector nucleic acid may be packaged into particles initially, and the particles used to deliver vector into the target cell population for selection. Subsequently, specific selection procedures to isolate virus variants possessing the desired phenotype are applied, and are described below.

In certain embodiments, random mutagenesis may be performed initially by propagation of the plasmid containing viral cDNA in the XL1-Red strain of *E. coli* (Stratagene, San Diego, CA), which is deficient in three of the primary DNA repair pathways, resulting from mutS, mutD, and mutT mutations. However, other mutagenesis procedures including, but not limited to, linker-scanning mutagenesis (Haltiner et al., *Nucleic Acids Res.* 13:1015, 1985; Barany, *Proc. Natl. Acad. Sci. USA*

82:4202, 1985), random oligonucleotide-directed mutagenesis (Kunkel et al., *Methods Enzymol.* 155:166, 1987; Zoller and Smith, *Methods Enzymol.* 154:329, 1987; Hill et al., *Methods Enzymol.* 155:558, 1987; Hermes et al., *Gene* 84:143, 1989) and PCR mutagenesis (Herlitze and Koenen, *Gene* 91:143, 1990), can be readily substituted  
5 utilizing published protocols. The resulting mixed population of mutated cDNA clones is introduced into susceptible cultured cells directly, or after transcription *in vitro*. Enrichment for transfected cells which contain mutated virus of the desired phenotype is accomplished based on increased survival time over wild-type virus infected cells, as described below.

10

### 3. Genetic Selection of Variants Using Virus-Derived Vectors

In another approach, mutations may be generated in any region a virus-derived expression vector, including the regulatory, untranslated regions, or protein-encoding gene regions. For example, within one aspect of the invention methods are  
15 provided for selecting viral variants with reduced or no inhibition of host-cell directed macromolecular synthesis, comprising the steps of: (a) introducing into a cell a eukaryotic layered vector initiation system, RNA vector replicon, or recombinant alphavirus particle which directs the expression of a immunogenic cell surface protein (suitable for detection of vector containing cells), or alternatively, a selectable marker  
20 (either a drug or non-drug marker wherein non-vector containing cells are killed upon addition of, for example, a drug such as neomycin, hygromycin, phleomycin, gpt, puromycin, or histidinol); (b) incubation or culturing the cells under conditions and for a time sufficient to select vector containing cells which exhibit the desired phenotype; followed by (c) isolating cells which contain the vector of the desired phenotype and (d)  
25 analysis of the vector for the causal mutation.

As noted above, the viral vectors of the present invention may be derived from a wide variety of viruses (e.g., Sindbis virus (Xiong et al., *Science* 243:1188-1191, 1989; Hahn et al., *Proc. Natl. Acad. Sci. USA* 89:2679-2683, 1992; Schlesinger, *Trends Biotechnol.* 11:18-22, 1993; Dubensky et al., *ibid*), Semliki Forest virus (Liljestrom  
30 and Garoff, *Bio/Technology* 9:1356-1361, 1991), Venezuelan equine encephalitis virus (Davis et al., *J. Cell. Biochem. Suppl.* 19A:310, 1995), poliovirus (Choi et al., *J. Virol.*

65:2875-2883, 1991; Ansardi et al., *Cancer Res.* 54:6359-6364, 1994; and Andino et al., *Science* 265:1448-1451, 1994). Representative examples of the above-described methods are discussed in more detail below within Example 2.

#### 5                      4.      Use of Viral Variants

As discussed in more detail herein, viral variants which have been selected or generated utilizing the methods provided herein may be utilized to construct a wide variety of recombinant gene delivery vehicles which exhibit the desired phenotype. Within certain embodiments, the gene delivery vehicle contains a mutation within the Leu-Xaa-Pro-Gly-Gly ("LXPGG") motif of the nsP2 gene. Briefly, for alphaviruses wherein published sequence of the nsP2 gene is available, a highly conserved amino acid motif -Leu-Xaa-Pro-Gly-Gly- ("LXPGG") is observed. As predicted by standard protein modelling algorithms (Chou and Fasman, *Adv. Enzym.* 47:45-148, 1978), the residues of this motif possibly comprise a  $\beta$  turn in the structure. Proline 726 of nsP2 in Sindbis virus is the central residue of this motif. The corresponding motif in other alphaviruses is illustrated in the table below.

<u>Alphavirus Strain*</u>	<u>Pro-Gly-Gly Region</u>	<u>nsP2 a.a.'s (P-G-G)</u>
1. Sindbis virus	Leu-Asn-Pro-Gly-Gly-Thr	a.a. = 726-728
2. S.A.AR86 virus	Leu-Asn-Pro-Gly-Gly-Thr	a.a. = 726-728
3. Ockelbo virus	Leu-Asn-Pro-Gly-Gly-Thr	a.a. = 726-728
4. Aura virus	Leu-Lys-Pro-Gly-Gly-Thr	a.a. = 725-727
5. Semliki Forest virus	Leu-Lys-Pro-Gly-Gly-Ile	a.a. = 718-720
6. VEE virus	Leu-Asn-Pro-Gly-Gly-Thr	a.a. = 713-715
7. Ross River virus	Leu-Xaa-Pro-Gly-Gly-Ser	a.a. = 717-719

\* Alphavirus strains with published nsP2 sequences: (1) Strauss et al., *Virology* 133:92-110, 1984; (2) Simpson et al., *Virology* 222:464-469 1996; (3) Shirako et al., *Virology* 182:753-764, 1991; (4) Rumenapf et al., *Virology* 208:621-633, 1995; (5) Takkinen, *Nucleic Acids Res.* 14:5667-5682, 1986; (6) Kinney et al., *Virology* 170:19-30, 1989; and (7) Faragher et al, *Virology* 163:509-526, 1988.

Hence, within various embodiments of the present invention, gene delivery vehicles are provided wherein the gene delivery vehicle contains an nsP2 gene with a mutation in the LXPGG motif. Within one embodiment, the Leu codon is

mutated to another amino acid selected from the group consisting of Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, or another rare or non-protein amino acid (*see, e.g.*, Lehninger, Biochemistry, Worth Publishers, Inc., N.Y. N.Y., 1975). Within another embodiment, the Pro codon is  
5 mutated to another amino acid selected from the group consisting of Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, Gly, His, Ile, Leu, Lys, Met, Phe, Ser, Thr, Trp, Tyr, Val, or another rare or non-protein amino acid. Within other embodiments, either or both of the Gly codons may be mutated to another amino acid selected from the group consisting of Ala, Arg, Asn, Asp, Asx, Cys, Gln, Glu, Glx, His, Ile, Leu, Lys, Met, Phe,  
10 Pro, Ser, Thr, Trp, Tyr, Val, or another rare or non-protein amino acid. Within yet other embodiments, the Xaa amino acid, or amino acids between 1 and 3 residues upstream or downstream of the LXPGG motif may be mutated from the wild-type amino acid in order to effect the phenotype of the resultant gene delivery vehicle. Within certain embodiments of the invention, the LXPGG motif may be mutated to contain more than  
15 one codon alteration, or alternatively, one or more codon insertions or deletions.

C. Alphavirus Vector Constructs and Alphavirus RNA Vector Replicons

As noted above, the present invention provides both DNA and RNA constructs which are derived from alphaviruses. Briefly, within one aspect of the  
20 present invention alphavirus vector constructs are provided, comprising a 5' promoter which initiates synthesis of viral RNA *in vitro* from cDNA, a 5' sequence which initiates transcription of alphavirus RNA, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins including an isolated nucleic acid molecule as described above, an alphavirus RNA polymerase recognition sequence and  
25 a 3' polyadenylate tract. Within other aspects, RNA vector replicons are provided, comprising a 5' sequence which initiates transcription of alphavirus RNA, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, including the isolated nucleic acid molecules discussed above, an alphavirus RNA polymerase recognition sequence and a 3' polyadenylate tract. Within preferred embodiments of the  
30 above, the above constructs further comprise a viral junction region. Each of these aspects are discussed in more detail below.

1. 5' Promoters which initiate synthesis of viral RNA

As noted above, within certain embodiments of the invention, alphavirus vector constructs are provided which contain 5' promoters which (e.g., DNA dependent RNA polymerase promoters) initiate synthesis of viral RNA from cDNA by a process of *in vitro* transcription. Within preferred embodiments such promoters include, for example, the bacteriophage T7, T3, and SP6 RNA polymerase promoters. Similarly, eukaryotic layered vector initiation systems are provided (e.g., DNA dependent RNA polymerase promoters) which contain 5' promoters which initiate synthesis of viral RNA from cDNA *in vivo* (i.e., within a cell). Within certain embodiments, such RNA polymerase promoters (for either alphavirus vector constructs or eukaryotic layered vector initiation systems) may be derived from both prokaryotic and eukaryotic organisms, and include, for example, the bacterial  $\beta$ -galactosidase and *trpE* promoters, and the eukaryotic viral simian virus 40 (SV40) (e.g., early or late), cytomegalovirus (CMV) (e.g., immediate early), Moloney murine leukemia virus (MoMLV) or Rous sarcoma virus (RSV) LTR, and herpes simplex virus (HSV) (thymidine kinase) promoters.

2. Sequences Which Initiate Transcription

As noted above, within preferred embodiments the alphavirus vector constructs and RNA vector replicons of the present invention contain a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also referred to as 5'-end CSE, or 5' *cis* replication sequence). Representative examples of such sequences include nucleotides 1-60, and to a lesser extent nucleotides through bases 150-210, of the wild-type Sindbis virus, nucleotides 10-75 for tRNA<sup>asp</sup> (aspartic acid, Schlesinger et al., U.S. Patent No. 5,091,309), and 5' sequences from other alphaviruses which initiate transcription. It is the complement of these sequences, which corresponds to the 3' end of the of the minus-strand genomic copy, which are bound by the nsP replicase complex, and possibly additional host cell factors, from which transcription of the positive-strand genomic RNA is initiated.

### 3. Alphavirus Nonstructural Proteins

The alphavirus vector constructs and RNA vector replicons provided herein also require sequences encoding all four alphaviral nonstructural proteins, including a sequence which provides the desired phenotype discussed above. Briefly, a wide variety of sequences which encode alphavirus nonstructural proteins, in addition to those explicitly provided herein, may be utilized in the present invention, and are therefore deemed to fall within the scope of the phrase "alphavirus nonstructural proteins." For example, due to the degeneracy of the genetic code, more than one codon may code for a given amino acid. Therefore, a wide variety of nucleic acid sequences which encode alphavirus nonstructural proteins may be generated. Furthermore, amino acid substitutions, additions, or deletions at any of numerous positions may still provide functional or biologically active nonstructural proteins. Within the context of the present invention, alphavirus nonstructural proteins are deemed to be biologically active if they promote self-replication of the vector construct, *i.e.*, replication of viral nucleic acids and not necessarily the production of infectious virus, and may be readily determined by metabolic labeling or RNase protection assays performed over a time course. Methods for making such derivatives are readily accomplished by one of ordinary skill in the art given the disclosure provided herein.

Alphaviruses express four nonstructural proteins, designated nsP1, nsP2, nsP3, and nsP4. Vectors of the present invention derived from alphaviruses should contain sequences encoding the four nonstructural proteins. In wild-type Sindbis virus, nonstructural proteins 1-3 are encoded by nucleotides 60 to 5747, while nsP4 is encoded by nucleotides 5769 to 7598 (*see* Figure 1). The nonstructural proteins are translated from the genomic positive strand RNA as one of two large polypeptides, known as P123 or P1234, respectively, depending upon (i) whether there is an opal termination codon between the coding regions of nsP3 and nsP4 and (ii) if there is such an opal codon present, whether there is translation termination of the nascent polypeptide at that point or readthrough and hence production of P1234. The opal termination codon is present at the nsP3/nsP4 junction of the alphaviruses SIN (strain AR339 and the SIN-1 strain described herein), AURA, WEE, EEE, VEE, and RR, and thus the P123 and P1234

species are expressed in cells infected with these viruses. In contrast, no termination codon is present at the nsP3/nsP4 junction of the alphaviruses SIN (strain AR86, SF, and ONN), and thus only the P1234 species is expressed in cells infected with these viruses. Both the polyprotein and processed monomeric forms of the nonstructural proteins function in the replication of the alphavirus RNA genome. Experiments examining growth characteristics of alphavirus nonstructural protein cleavage mutants have indicated that the polyproteins are involved in the synthesis of the genomic negative stranded RNA, while the individual monomeric proteins catalyze the synthesis of the genomic and subgenomic positive stranded RNA species (Shirako and Strauss, *J. Virol.* 68:1874-1885, 1994). Translational readthrough generally occurs about 10%-20% of the time in cells infected with wild type Sindbis virus containing the opal termination codon at the nsP3/nsP4 junction. Processing of P123 and P1234 is by a proteinase activity encoded by the one of the nonstructural proteins, and is discussed further below. The order of processing, whether in *cis* or in *trans*, depends on various factors, including the stage of infection. For example, Sindbis virus and SFV produce P123 and nsP4 early in infection, and P12 and P34 later in infection. Further processing then releases the individual nonstructural proteins. Each nonstructural protein has several functions, some of which are described below.

20                   a.     nsP1

Nonstructural protein 1 is required for the initiation of (or continuation of) minus-strand RNA synthesis. It also plays a role in capping the 5' terminus of genomic and subgenomic alphavirus RNAs during transcription, as nsP1 possesses both methyltransferase (Mi and Stollar, *Vir.* 184:423-427, 1991) and guanylttransferase activity (Strauss and Strauss, *Microbiol. Rev.* 58(3):491-562, 1994). NsP1 also modulates the proteinase activity of nsP2, as polyproteins containing nsP1 inefficiently cleave between nsP2 and nsP3 (de Groot et al., *EMBO J.* 9:2631-2638, 1990).

                  b.     nsP2

30                   Nonstructural protein 2 is a multifunctional protein, involved in the replication of the viral RNA and processing of the nonstructural polyprotein. The N-



terminal domain of the protein (spanning about the first 460 amino acids) is believed to be a helicase which is active in duplex unwinding during RNA replication and transcription. Synthesis of 26S subgenomic mRNA, which, in vectors according to the present invention, encodes the gene(s) of interest, requires functional nsP2. The C-terminal domain of nsP2, between amino acid residues 460-807 of Sindbis virus, proteolytically cleaves in *trans* and in *cis* the nonstructural polyprotein between the nsP1/nsP2, nsP2/nsP3, and nsP3/nsP4 junctions. Alignment of the primary sequences of the alphavirus nsP2 C-terminal domains suggests that nsP2 is a papain-like proteinase (Hardy and Strauss, *J. Virol.* 63:4653-4664, 1988).

Other observed characteristics of nsP2 have not, as yet, been assigned a function directly related to the propagation of alphaviruses. For example, it has been shown that nsP2 is closely associated with ribosomes in SFV-infected cells, and can be cross-linked to rRNA by UV irradiation (Ranki et al., *FEBS Lett.* 108:299-302, 1979). Further, 50% of nsP2 is localized in the nuclear matrix, particularly in the area of the nucleoli of SFV-infected BHK cells (Peränen et al., *J. Virol.* 64:1888-1896, 1990). Localization of nsP2 to the nuclei presumably proceeds by active transport, as it exceeds the size of small proteins and metabolites (about 20-60 kD), which can enter the nucleus by diffusion through nuclear core complexes (Paine et al., *Nature* 254:109-114, 1975). Putative NLS sequences have been identified in the alphaviruses SFV, SIN, RR, ONN, OCK, and VEE (Rikkonen et al., *Vir.* 189:462-473, 1992).

#### c. nsP3

Nonstructural protein nsP3 contains two distinct domains, although their precise roles in viral replication are not well understood. The N-terminal domain ranges in length from 322 to 329 residues in different alphaviruses and exhibits a minimum of 51% amino acid sequence identity among any two alphaviruses. The C-terminal domain, however, is not conserved among known alphaviruses in length or in sequence, and multiple changes are tolerated (Li et al., *Virology*, 179:416-427). The protein is found associated with replication complexes in a heavily phosphorylated state. In alphaviruses whose genomes contain an opal termination codon between the nsP3/nsP4 junction, two different proteins are produced depending upon whether or not there is

readthrough of the opal termination signal. Readthrough results in an nsP3 protein which contains 7 additional carboxy terminal amino acids after cleavage of the polyprotein. It is clear that nsP3 is required in some capacity for viral RNA synthesis, as particular mutants of this protein are RNA negative, and the P123 polyprotein is  
5 required for minus-strand RNA synthesis.

d. nsP4

NsP4 is the virus-encoded RNA polymerase and contains the GDD motif characteristic of such enzymes (Kamer and Argos, *Nucleic Acids Res.* 12:7269-7282,  
10 1984). Thus, nsP4 is indispensable for alphavirus RNA replication. The concentration of nsP4 is tightly regulated in infected cells. In most alphaviruses, translation of nsP4 requires readthrough of an opal codon between the nsP3 and nsP4 coding regions, resulting in lower intracellular levels as compared to other nonstructural proteins. Additionally, the bulk of nsP4 is metabolically unstable, through degradation by the N-  
15 end rule pathway (Gonda et al., *J. Biol. Chem.* 264:16700-16712, 1989). However, some nsP4 is stable, due to its association with replication complexes which conceal degradation signals. Thus, stabilization of the enzyme by altering the amino terminal residue may prove useful in promoting more long term expression of proteins encoded by the vectors described herein. Stabilizing amino terminal residues include  
20 methionine, alanine, and tyrosine.

4. Viral Junction Regions

The alphavirus viral junction region normally controls transcription initiation of the subgenomic mRNA; thus, this element is also referred to as the  
25 subgenomic mRNA promoter. In the case of Sindbis virus, the normal viral junction region typically begins at approximately nucleotide number 7579 and continues through at least nucleotide number 7612 (and possibly beyond). At a minimum, nucleotides 7579 to 7602 (5'- ATC TCT ACG GTG GTC CTA AAT AGT - SEQ. ID NO. 1) are believed necessary for transcription of the subgenomic fragment. This region  
30 (nucleotides 7579 to 7602) is hereinafter referred to as the "minimal junction region core."

Within certain aspects of the invention, the viral junction region is inactivated in order to prevent synthesis of the subgenomic fragment. As utilized within the context of the present invention, "inactivated" means that the species corresponding to subgenomic mRNA is not observed in autoradiograms from  
5 denaturing gels of electrophoresed RNA purified from cells containing these vectors and treated with 1 µg/ml dactinomycin and labeled with [<sup>3</sup>H]-uridine, as described (Frolov and Schlesinger, *J. Virol.* 68:1721-1727, 1994).

Within one embodiment of the invention, gene delivery vehicles may be constructed by the placement of signals promoting either ribosome readthrough or  
10 internal ribosome entry immediately downstream of the disabled junction region promoter. In this vector configuration, synthesis of subgenomic message cannot occur; however, the heterologous proteins are expressed from genomic length mRNA by either ribosomal readthrough (scanning) or internal ribosome entry.

In certain applications of the gene delivery vehicles described herein, the  
15 expression of more than one heterologous gene is desired. For example, in order to treat metabolic disorders such as Gaucher's syndrome, multiple administrations of gene delivery vehicles or particles may be required, since duration of the therapeutic palliative may be limited. Therefore, within certain embodiments of the invention it may be desirable to co-express in a target cell the Adenovirus 2 E3 gene, along with a  
20 therapeutic palliative, such as the glucocerebrosidase gene. In wild-type virus, the structural protein (sP) polycistronic message is translated into a single polyprotein which is processed subsequently into individual proteins in part by the sP capsid proteinase. Thus, expression of multiple heterologous genes from a polycistronic message requires a mechanism different from the wild-type virus, since the protease  
25 activity of the capsid sP, or the peptides recognized for cleavage, are not present in the replacement region of the alphavirus vectors. Therefore, within further embodiments of the invention, functional elements which permit translation of multiple independent heterologous sequences, including ribosomal readthrough, cap-independent translation, internal ribosome entry, or minimal junction region core sequences, can be utilized.

5. Alphavirus RNA polymerase recognition sequence, and poly(A) tract

As noted above, alphavirus vector constructs or RNA vector replicons of the present invention also should include an alphavirus RNA polymerase recognition sequence (also termed "alphavirus replicase recognition sequence", "3' terminal CSE", or "3' *cis* replication sequence"). Briefly, the alphavirus RNA polymerase recognition sequence, which is located at the 3' end region of positive stranded genomic RNA, provides a recognition site at which the virus begins replication by synthesis of the negative strand. A wide variety of sequences may be utilized as an alphavirus RNA polymerase recognition sequence. For example, within one embodiment, Sindbis virus vector constructs in which the polymerase recognition is truncated to the smallest region that can still function as a recognition sequence (*e.g.*, nucleotides 11,684 to 11,703) can be utilized. Within another embodiment of the invention, Sindbis virus vector constructs in which the entire nontranslated region downstream from the E1 sP gene to the 3' end of the viral genome including the polymerase recognition site (*e.g.*, nucleotides 11,382 to 11,703), can be utilized.

Within preferred embodiments of the invention, the alphavirus vector construct or RNA vector replicon may additionally contain a poly(A) tract, which increases dramatically the observed level of heterologous gene expression in cells transfected with alphavirus-derived vectors (*see e.g.*, Dubensky et al, *supra*). Briefly, the poly(A) tract may be of any size which is sufficient to promote stability in the cytoplasm, thereby increasing the efficiency of initiating the viral life cycle. Within various embodiments of the invention, the poly(A) sequence comprises at least 10 adenosine nucleotides, and most preferably, at least 25 adenosine nucleotides. Within one embodiment, the poly(A) sequence is attached directly to Sindbis virus nucleotide 11,703.

D. Eukaryotic Layered Vector Initiation Systems

Due to the size of a full-length genomic alphavirus cDNA clone, *in vitro* transcription of full-length, capped RNA molecules is rather inefficient. This results in a lowered transfection efficiency, in terms of infectious centers of virus (as measured by plaque formation), relative to the amount of *in vitro* transcribed RNA transfected. Such

inefficiency is also relevant to the *in vitro* transcription of alphavirus expression vectors. Testing of candidate cDNA clones and other alphavirus cDNA expression vectors for their ability to initiate an infectious cycle or to direct the expression of a heterologous sequence can thus be greatly facilitated if a cDNA clone is transfected into  
5 susceptible cells as a DNA molecule, which then directs the synthesis of viral RNA *in vivo*.

Therefore, within one aspect of the present invention DNA-based vectors (referred to as "Eukaryotic Layered Vector Initiation Systems") are provided that are capable of directing the synthesis of viral RNA (genomic or vector) *in vivo*. Generally,  
10 eukaryotic layered vector initiation systems comprise a 5' promoter that is capable of initiating *in vivo* (*i.e.*, within a cell) the 5' synthesis of RNA from cDNA, a construct that is capable of directing its own replication in a cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence that controls transcription termination (*e.g.*, a polyadenylate tract). Such eukaryotic layered vector  
15 initiation systems provide a two-stage or "layered" mechanism that controls expression of heterologous nucleotide sequences. Briefly, the first layer initiates transcription of the second layer and comprises a promoter that is capable of initiating *in vivo* the 5' to 3' synthesis of RNA from cDNA (*e.g.*, a 5' eukaryotic promoter), and may further comprise other elements, including a 3' transcription termination/polyadenylation site,  
20 one or more splice sites, as well as other RNA nuclear export elements, including, for example, the hepatitis B virus posttranscriptional regulatory element (PRE) (Huang et al., *Mol. Cell. Biol.* 13:7476, 1993; Huang et al., *J. Virol.* 68:3193, 1994; Huang et al., *Mol. Cell. Biol.*, 15:3864-3869, 1995), the Mason-Pfizer monkey virus constitutive transport element (CTE) (Bray et al., *Proc. Natl. Acad. Sci. USA* 91:1256-1260, 1994),  
25 the HIV Rev responsive element (Malim et al., *Nature* 338:254-257, 1989; Cullen et al., *Trends Biochem. Sci.* 16:346, 1991), and other similar elements, if desired. Representative promoters suitable for use within the present invention include both eukaryotic (*e.g.*, pol I, II, or III) and prokaryotic promoters, and inducible or non-inducible (*i.e.*, constitutive) promoters, such as, for example, Moloney murine leukemia  
30 virus promoters, metallothionein promoters, the glucocorticoid promoter, *Drosophila*

actin 5C distal promoter, SV40 promoter, heat shock protein 65 promoter, heat shock protein 70 promoter, immunoglobulin promoters, mouse polyoma virus promoter (Py), Rous sarcoma Virus (RSV), herpes simplex virus (HSV) promoter, BK virus and JC virus promoters, mouse mammary tumor virus (MMTV) promoter, alphavirus junction  
5 region, CMV promoter, Adenovirus E1 or VA1RNA promoters, rRNA promoters, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, tetracycline responsive promoter, and the *lac* promoter.

Within yet other embodiments of the invention, inducible promoters may be utilized. For example, within one embodiment inducible promoters are provided  
10 which initiate the synthesis of RNA from DNA, comprising a core RNA polymerase promoter sequence, and an operably linked nucleic acid sequence that directs the DNA binding of a transcriptional activator protein, and an operably linked nucleic acid sequence that directs the DNA binding of a transcriptional repressor protein. Within a further embodiment, the nucleic acid sequence that directs the DNA binding of a  
15 transcriptional activator protein is a sequence that binds a tetracycline repressor/VP16 transactivator fusion protein. Within yet another embodiment, the nucleic acid sequence that directs the DNA binding of a transcription repressor protein is a sequence that binds a lactose repressor / Kruppel domain fusion protein.

The second layer comprises an autocatalytic vector construct which is  
20 capable of expressing one or more heterologous nucleotide sequences and of directing its own replication in a cell, either autonomously or in response to one or more factors (e.g. is inducible). The second layer may be of viral or non-viral origin. Within one embodiment of the invention, the second layer construct may be an alphavirus vector construct as described above.

25 A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al.,  
30 *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330 and 5,017,487; WO

89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., *Nature* 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., *J. Vir.* 63:3822-3828, 1989; Mendelson et al., *Virol.* 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, *Adv. Exp. Med. Biol.* 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, *J. Virol.* 65:532-536, 1991).

Similarly, a wide variety of vector systems may be utilized as second layer of the eukaryotic layered vector initiation system, including for example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g., alphavirus, rubella), Flaviviridae (e.g., yellow fever, HCV), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta virus and Astrovirus. In addition, non-mammalian RNA viruses (as well as components derived therefrom) may also be utilized, including for example, bacterial and bacteriophage replicases, as well as components derived from plant viruses, such as potexviruses (e.g., PVX), carlaviruses (e.g., PVM), tobnaviruses (e.g., TRV, PEBV, PRV), Tobamoviruses (e.g., TMV, ToMV, PPMV), luteoviruses (e.g., PLRV), potyviruses (e.g., TEV, PPV, PVY), tombusviruses (e.g., CyRSV), nepoviruses (e.g., GFLV), bromoviruses (e.g., BMV), and topamoviruses.

The replication competency of the autocatalytic vector construct, contained within the second layer of the eukaryotic vector initiation system, may be measured by a variety of assays known to one of skill in the art including, for example, ribonuclease protection assays which measure increases of both positive-sense and negative-sense RNA in transfected cells over time, in the presence of an inhibitor of cellular RNA synthesis, such as dactinomycin, and also assays which measure the

synthesis of a subgenomic RNA or expression of a heterologous reporter gene in transfected cells.

Within particularly preferred embodiments of the invention, eukaryotic layered vector initiation systems are provided that comprise a 5' promoter which is  
5 capable of initiating *in vivo* the synthesis of alphavirus RNA from cDNA (*i.e.*, a DNA promoter of RNA synthesis), followed by a 5' sequence which is capable of initiating transcription of an alphavirus RNA, a nucleic acid sequence which operably encodes all four alphaviral nonstructural proteins (including a nucleic acid molecule as described above which, when operably incorporated into a recombinant alphavirus particle, results  
10 in the desired phenotype), an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination/polyadenylation. In addition, a viral junction region which is operably linked to a heterologous sequence to be expressed may be included. Within various embodiments, the viral junction region may be modified, such that viral transcription of the subgenomic fragment is increased or  
15 reduced, rather than inactivated. Within other embodiments, a second viral junction region may be inserted following the first inactivated viral junction region, the second viral junction region being either active or modified such that viral transcription of the subgenomic fragment is increased or reduced.

Following transcription of the eukaryotic layered vector initiation  
20 system, the resulting alphavirus RNA vector replicon molecule is comprised of a 5' sequence which is capable of initiating transcription of an alphavirus RNA, a nucleotide sequence encoding biologically active alphavirus nonstructural proteins, a viral junction region, a heterologous nucleotide sequence, an alphavirus RNA polymerase recognition sequence, and a polyadenylate sequence.

25 Various aspects of the alphavirus cDNA vector constructs have been discussed above, including the 5' sequence which is capable of initiating transcription of an alphavirus, the nucleotide sequence encoding alphavirus nonstructural proteins, the viral junction region, including junction regions which have been inactivated such that viral transcription of the subgenomic fragment is prevented, and the alphavirus RNA



polymerase recognition sequence. In addition, modified junction regions and tandem junction regions have also been discussed above.

In another embodiment of the invention, the eukaryotic layered vector initiation system is derived from an alphavirus vector, such as a Sindbis vector  
5 construct, which has been adapted to replicate in one or more cell lines from a particular eukaryotic species, especially a mammalian species, such as humans. For instance, if the gene encoding the recombinant protein to be expressed is of human origin and the protein is intended for human therapeutic use, production in a suitable human cell line may be preferred in order that the protein be post-translationally modified as would be  
10 expected to occur in humans. This approach may be useful in further enhancing recombinant protein production (as discussed in more detail below). Given the overall plasticity of an alphaviral genome due to the infidelity of the viral replicase, variant strains with an enhanced ability to establish high titer productive infection in selected eukaryotic cells (*e.g.*, human, murine, canine, feline, etc.) can be isolated. Additionally,  
15 variant alphaviral strains having an enhanced ability to establish high titer persistent infection in eukaryotic cells may also be isolated using this approach. Alphavirus expression vectors can then be constructed from cDNA clones of these variant strains according to procedures provided herein.

Within another embodiment of the invention, the eukaryotic layered  
20 vector initiation system comprises a promoter for initial alphaviral vector transcription that is transcriptionally active only in a differentiated cell type. Briefly, it is well established that alphaviral infection of mammalian cells in culture, such as those derived from hamster (*e.g.*, baby hamster kidney cells) or chicken (*e.g.*, chicken embryo fibroblasts), typically results in cytotoxicity. Thus, to produce a stably transformed or  
25 transfected host cell line, the eukaryotic layered vector initiation system may be introduced into a host cell wherein the promoter which enables the initial vector amplification is a transcriptionally inactive, but inducible, promoter. In a particularly preferred embodiment, such a promoter is differentiation state dependent. In this configuration, activation of the promoter and subsequent activation of the alphavirus  
30 DNA vector coincides with induction of cell differentiation. Upon growth to a certain

cell number of such a stably transformed or transfected host cell line, the appropriate differentiation stimulus is provided, thereby initiating transcription of the vector construct and amplified expression of the desired gene and encoded polypeptide(s). Many such differentiation state-dependent promoters are known to those in the art, as  
5 are cell lines which can be induced to differentiate by application of a specific stimulus. Representative examples include cell lines F9 and P19, HL60, and Freund erythroleukemic cell lines and HEL, which are activated by retinoic acid, horse serum, and DMSO, respectively.

In a preferred embodiment, such promoters can be regulated by two  
10 separate components. For example, as described in Example 7, binding sites for both a transcriptional activator and a transcriptional repressor are positioned adjacent to a "core" promoter, in an operably-dependent manner. In this configuration, the uninduced state is maintained by blocking the ability of the transcriptional activator to find its recognition site, while allowing the transcriptional repressor to be constitutively  
15 expressed and bound to its recognition site. Induction is permitted by blocking the transcriptional repressor and removing the transactivator block. For example, a tetracycline-responsive promoter system (Gossen and Bujard, *Proc. Natl. Acad. Sci.* 89:5547-5551, 1992) may be utilized for inducible transcription of an alphavirus vector RNA. In this system, the expression of a tetracycline repressor and HSV-VP16  
20 transactivator domain, as a "fusion" protein (rTA), stimulates *in vivo* transcription of the alphavirus vector RNA by binding specifically to a tetracycline operator sequence (tetO) located immediately adjacent to a minimal "core" promoter (for example, CMV). The binding and transactivation event is reversibly blocked by the presence of tetracycline, and may be "turned on" by removing tetracycline from the culture media.  
25 As uninduced basal levels of transcription will vary among different cell types, other different minimal core promoters (for example HSV-tk) may be linked to the tetracycline operator sequences, provided the transcription start site is known, to allow juxtaposition at or in the immediate proximity of alphavirus vector nucleotide 1.

The rTA transactivator can be provided by an additional expression  
30 cassette also stably transformed into the same cell line; and in certain embodiments, the

rTA expression cassette may itself be autoregulatory. The use of an autoregulatory rTA expression cassette circumvents potential toxicity problems associated with constitutive high level expression of rTA by linking expression to transcriptional control by the same tetO-linked promoter to which rTA itself binds. This type of system creates a negative feedback cycle that ensures very little rTA is produced in the presence of tetracycline, but becomes highly active when the tetracycline is removed. Such an autoregulatory rTA expression cassette is provided in plasmid pTet-tTAk (Shockett et al., *Proc. Natl. Acad. Sci. USA* 92:6522-6526, 1995).

For transcriptional repression, the KRAB repression domain of a certain zinc finger proteins can also be utilized. Briefly, KRAB (Krüppel-associated box) domains are highly conserved sequences present in the amino-terminal regions of more than one-third of all Krüppel-class Cys<sub>2</sub>His<sub>2</sub> zinc finger proteins. The domains contain two predicted amphipathic  $\alpha$ -helicies and have been shown to function as DNA binding-dependent RNA polymerase II transcriptional repressors (for example, Licht et al., *Nature* 346: 76-79, 1990). Like other transcription factors, the active repression domain and the DNA-binding domain are distinct and separable. Therefore, the repression domain can be linked as a fusion protein to any sequence specific DNA binding protein for targeting. Thus, the DNA binding protein component can be reversibly prevented from binding in a regulatable fashion, thereby turning "off" the transcriptional silencing. For example, the KRAB domain from human Kox1 (Thiesen, *New Biol.* 2:363-374, 1990) can be fused to the DNA-binding lactose (lac) repressor protein, forming a hybrid transcriptional silencer with reversible, sequence-specific binding to a lac operator sequence engineered immediately adjacent to the tet-responsive promoter. In this configuration, constitutive expression of the lac repressor/KRAB domain fusion (rKR) will result in binding to the lac operator sequence and the elimination of any "leaky" basal transcription from the uninduced tet-responsive promoter. When vector expression is desired and tetracycline is removed from the system, IPTG is added to prevent rKR-mediated transcriptional silencing.

In addition, KRAB domains from other zinc finger proteins, for example, ZNF133 (Tommerup et al., *Hum. Mol. Genet.* 2:1571-1575, 1993), ZNF91 (Bellefroid

et al., *EMBO J.* 12:1363-1374, 1993), ZNF2 (Rosati et al., *Nucleic Acids Res.* 19:5661-5667, 1991), as well as other transferable repressor domains, for example, *Drosophila en* or *eve* genes (Jaynes and O'Farrell, *EMBO J.* 10:1427-1433, 1991; Han and Manley, *Genes Dev.* 7:491-503, 1993), human zinc finger protein YY1 (Shi et al., *Cell* 67:377-388, 1991), Wilms' tumor suppressor protein WT1 (Madden et al., *Science* 253:1550-1553, 1991), thyroid hormone receptor (Baniahmad et al., *EMBO J.* 11:1015-1023, 1992), retinoic acid receptor (Baniahmad et al., *ibid*), Kid-1 (Witzgall et al., *Proc. Natl. Acad. Sci. USA* 91:4514-4518, 1994), can likewise be readily used in the gene delivery vehicles provided herein. Furthermore, the lac repressor/lac operator component of this system may be substituted by any number of other regulatable systems derived from other sources, for example, the tryptophan and maltose operons, or GAL4.

E. Recombinant Alphavirus Particles, and Generation and Use of 'Empty' Togavirus Particles or Togaviruses Particles containing non-homologous viral RNA

Within another aspect of the present invention, the generation of recombinant alphavirus particles containing RNA alphavirus vectors, which are capable of infection of eukaryotic target cells, are described. Briefly, such recombinant alphavirus particles generally comprise one or more alphavirus structural proteins, a lipid envelope, and an RNA vector replicon as described herein.

Methods for generating recombinant alphavirus vector particles may be readily accomplished by, for example, co-transfection of complementing vector and defective helper (DH) molecules derived from *in vitro* transcribed RNA, or, alternatively, plasmid DNA, or by coinfection with virus (see Xiong et al., *Science* 243:1188-1191, 1989, Bredenbeek et al., *J. Virol.* 67:6439-6446, 1993, Dubensky et al., *J. Virol* 70:508-519, 1996 and Dubensky et al., W/O 95/07994).

Within other aspects, methods for generating recombinant alphavirus vector particles from alphavirus-derived packaging or producer cell lines are provided. Briefly, such PCL and their stably transformed structural protein expression cassettes can be derived using methods described within W/O 95/07994, or using novel methods

described within this invention. For example, the production of recombinant alphavirus vector particles by PCL can be accomplished following introduction of alphavirus-based vector molecules with desirable properties into the PCL (*see* Example 6), the vectors being derived from *in vitro* transcribed RNA, plasmid DNA, or previously  
5 obtained recombinant alphavirus particles. In yet a further example, production of recombinant particles from alphavirus vector producer cell lines is described (*see* Example 7).

Within other embodiments, methods are provided for producing high-titer stable togavirus capsid particles that do not contain any genomic RNA (*i.e.*, contain  
10 substantially no viral RNA) or RNA Vector Replicons. As utilized within the present invention, it should be understood that "substantially no" genomic or RNA Vector Replicon nucleic acids refers to ratios of greater than 10:1, and preferably greater than 15:1 of <sup>35</sup>S methionine versus <sup>3</sup>H uridine incorporation into virus particles (as compared to wild-type) (*see, e.g.*, Example 8 and Figure 38). For example, within one  
15 embodiment empty capsid particles (preferably with the lipid bilayer and glycoprotein complement) are constructed from a selected pathogenic virus from the togavirus family (such as an Alphavirus or Rubivirus), and used as immunogens to establish protective immunity against infection with the wild-type togavirus. The empty viral particles are a desirable immunogenic alternative, as they are unable to replicate and produce virus, yet  
20 are able to generate both cellular and humoral immune responses. Thus, utilizing the methods which are described in more detail in Example 8, empty capsid particles derived from togaviruses (with or without a lipid bilayer and glycoprotein complement) can be generated from a wide variety of togaviruses, including, but not limited to, alphaviruses (such as Sindbis Virus (*e.g.*, SIN-1 or wild-type Sindbis virus),  
25 Venezuelan Equine Encephalitis virus, Ross River virus, Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, and rubiviruses (*e.g.*, rubella),

In a second embodiment, sequences from heterologous viruses which encode peptides that bind to genomic viral RNA can be inserted into a defective helper (DH) expression cassette in the amino terminal region of the alphavirus capsid gene,  
30 which has been deleted of the sequences which encode the region of the protein that

binds to the homologous alphavirus genomic RNA. For example, BHK cells can be electroporated with an alphavirus replicon RNA, a DH RNA containing a sequence that encodes a heterologous virus genomic RNA binding peptide, and a replicon derived from the same heterologous virus. Thus, the alphavirus particles produced contain a  
5 genomic RNA from a heterologous virus, and possess the host-range tropism of the alphavirus. As one possible example, *gag* sequences encoding proteins required for retrovirus RNA binding are included in the DH expression cassette construct. In this configuration, the resulting alphavirus particles would contain retrovirus vector RNA.

10 F. Heterologous Sequences

As noted above, a wide variety of nucleotide sequences may be carried and expressed by the gene delivery vehicles of the present invention. Preferably, the nucleotide sequences should be of a size sufficient to allow production of viable virus. Within the context of the present invention, the production of any measurable titer by  
15 recombinant alphavirus particles, for example, by plaque assay, luciferase assay, or  $\beta$ -galactosidase assay of infectious virus on appropriate susceptible monolayers, or the expression of detectable levels of the heterologous gene product by RNA or DNA vectors, is considered to be "production of viable virus." This may be, at a minimum, an alphavirus vector construct which does not contain any additional heterologous  
20 sequence. However, within other embodiments, the vector construct may contain additional heterologous or foreign sequences. Within preferred embodiments, the heterologous sequence can comprise a heterologous sequence of at least about 100 bases, 2 kb, 3.5 kb, 5 kb, 7 kb, or even a heterologous sequence of at least about 8 kb.

As will be evident to one of ordinary skill in the art given the disclosure  
25 provided herein, the efficiency of recombinant alphavirus particle packaging and hence, viral titer, is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production of viable virus, additional non-coding sequences may be added to the vector construct. Moreover, within certain embodiments of the invention it may be desired to increase or decrease  
30 viral titer. This increase or decrease may be accomplished by increasing or decreasing the size of the heterologous sequence, and hence the efficiency of packaging.

As briefly noted above, a wide variety of heterologous sequences may be included within the gene delivery vehicles described herein including, for example, sequences which encode palliatives such as lymphokines or cytokines, toxins, prodrug converting enzyme, antigens which stimulate an immune response, ribozymes, proteins  
5 for therapeutic application such as growth or regulatory factors, and proteins which assist or inhibit an immune response, as well as antisense sequences (or sense sequences for "antisense applications"). In addition, as discussed above, the gene delivery vehicles provided herein may contain (and express, within certain embodiments) two or more heterologous sequences.

10

1. Lymphokines

Within one embodiment of the invention, the heterologous sequence encodes a lymphokine. Briefly, lymphokines act to proliferate, activate, or differentiate immune effectors cells. Representative examples of lymphokines include gamma  
15 interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, CSF-1 and G-CSF.

Within related embodiments of the invention, the heterologous sequence encodes an immunomodulatory cofactor. Briefly, as utilized within the context of the present invention, "immunomodulatory cofactor" refers to factors which, when  
20 manufactured by one or more of the cells involved in an immune response, or when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the cofactor. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular  
25 proliferation (e.g.,  $^3\text{H}$  thymidine uptake), and *in vitro* cytotoxic assays (e.g., which measure  $^{51}\text{Cr}$  release) (see Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991).

Representative examples of immunomodulatory co-factors include alpha interferon (Finter et al., *Drugs* 42(5):749-765, 1991; U.S. Patent No. 4,892,743; U.S.  
30 Patent No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz.* 78:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci.*

USA 86:2046-2050, 1989; Faktor et al., *Oncogene* 5:867-872, 1990), beta interferon (Seif et al., *J. Virol.* 65:664-671, 1991), gamma interferons (Radford et al., *American Society of Hepatology*:2008-2015, 1991; Watanabe et al., *PNAS* 86:9456-9460, 1989; Gansbacher et al., *Cancer Research* 50:7820-7825, 1990; Maio et al., *Can. Immunol.* 5 *Immunother.* 30:34-42, 1989; U.S. Patent Nos. 4,762,791 and 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), TNFs (Jayaraman et al., *J. Immunology* 144:942-951, 1990), Interleukin- 2 (IL-2) (Karupiah et al., *J. Immunology* 144:290-298, 1990; Weber et al., *J. Exp. Med.* 166:1716-1733, 1987; Gansbacher et al., *J. Exp. Med.* 172:1217-1224, 1990; U.S. Patent No. 4,738,927), IL-4 10 (Tepper et al., *Cell* 57:503-512, 1989; Golumbek et al., *Science* 254:713-716, 1991; U.S. Patent No. 5,017,691), IL-6 (Brakenhof et al., *J. Immunol.* 139:4116-4121, 1987; WO 90/06370), IL-12, IL-15 (Grabstein et al., *Science* 264:965-968, 1994; Genbank-EMBL Accession No. V03099), ICAM-1 (Altman et al., *Nature* 338:512-514, 1989), ICAM-2, LFA-1, LFA-3, MHC class I molecules, MHC class II molecules, 15  $\alpha_2$ -microglobulin, chaperones, CD3, B7/BB1, MHC linked transporter proteins or analogues thereof.

The choice of which immunomodulatory cofactor to include within a alphavirus vector construct may be based upon known therapeutic effects of the cofactor, or experimentally determined. For example, in chronic hepatitis B infections 20 alpha interferon has been found to be efficacious in compensating a patient's immunological deficit and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory cofactor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated *in vitro* with autologous or HLA-matched cells 25 (e.g., EBV transformed cells), and transduced with an alphavirus vector construct which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory cofactor. Stimulated PBLs are used as effectors in a CTL assay with the HLA-matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA-matched stimulator and target cells 30 transduced with a vector encoding the antigen alone, indicates a useful



immunomodulatory cofactor. Within one embodiment of the invention, the immunomodulatory cofactor gamma interferon is particularly preferred.

Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. The second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., *J. Exp. Med.* 173:721-730, 1991a, and *J. Exp. Med.* 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8<sup>+</sup> T cells, such that the CD8<sup>+</sup> T cells produce enough IL-2 to expand and become fully activated. These CD8<sup>+</sup> T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB1 factor and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen-presenting cells. The HBV core-specific CTL response will be augmented from the fully activated CD8<sup>+</sup> T cell via the costimulatory ligand B7/BB1.

## 2. Toxins

Within another embodiment of the invention, the heterologous sequence encodes a toxin. Briefly, toxins act to directly inhibit the growth of a cell. Representative examples of toxins include ricin (Lamb et al., *Eur. J. Biochem.* 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem.* 198:723-732, 1991; Evensen et al., *J. of Biol. Chem.* 266:6848-6852, 1991; Collins et al., *J. of Biol. Chem.* 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc.* 309:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem.* 260:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature* 306:551-557, 1983; Sanchez and Holmgren, *PNAS* 86:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem.* 255:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther.*

21:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J.* 203:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys.* 200:418-425, 1980; Irvin, *Arch. Biochem. & Biophys.* 169:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS* 84:4364-4368, 1987; Jackson et al., *Microb. Path.* 2:147-153, 1987), Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem.* 262:8707-8711, 1987), herpes simplex virus thymidine kinase (HSVTK) (Field et al., *J. Gen. Virol.* 49:115-124, 1980), and *E. coli* guanine phosphoribosyl transferase.

### 3. Prodrug converting enzymes

10           Within other embodiments of the invention, the heterologous sequence encodes a prodrug converting enzyme. Briefly, as utilized within the context of the present invention, a prodrug converting enzyme refers to a gene product that activates a compound with little or no cytotoxicity into a toxic product (the prodrug). Representative examples of such gene products include HSVTK and VZVTK (as well  
15 as analogues and derivatives thereof), which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs ganciclovir, acyclovir, or any of their analogues (e.g., FIAU, FIAC, DHPG) to HSVTK phosphorylates the drug into its corresponding active nucleotide triphosphate form.

20           Representative examples of other prodrug converting enzymes which can also be utilized within the context of the present invention include: *E. coli* guanine phosphoribosyl transferase which converts thioxanthine into toxic thioxanthine monophosphate (Besnard et al., *Mol. Cell. Biol.* 7:4139-4141, 1987); alkaline phosphatase, which converts inactive phosphorylated compounds such as mitomycin  
25 phosphate and doxorubicin-phosphate into toxic dephosphorylated compounds; fungal (e.g., *Fusarium oxysporum*) or bacterial cytosine deaminase, which converts 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS* 89:33, 1992); carboxypeptidase G2, which cleaves the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and  
30 Penicillin-V amidase, which converts phenoxyacetamide derivatives of doxorubicin and

melphalan to toxic compounds (see generally, Vrudhula et al., *J. of Med. Chem.* 36(7):919-923, 1993; Kern et al., *Canc. Immun. Immunother.* 31(4):202-206, 1990).

#### 4. Antisense Sequences

5           Within another embodiment of the invention, the heterologous sequence is an antisense sequence. Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein or prevent use of that RNA sequence by the cell. Representative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick,  
10   *Arch. Biochem. & Biophys.* 253:214-220, 1987; Bzik et al., *PNAS* 84:8360-8364, 1987), antisense HER2 (Coussens et al., *Science* 230:1132-1139, 1985), antisense ABL (Fainstein et al., *Oncogene* 4:1477-1481, 1989), antisense Myc (Stanton et al., *Nature* 310:423-425, 1984) and antisense *ras*, as well as antisense sequences which block any of the cell cycle signaling components (e.g., cyclins, cyclin-dependent kinases, cyclin-  
15   dependent kinase inhibitors) or enzymes in the nucleotide biosynthetic pathway. In addition, within other embodiments of the invention antisense sequences to interferon and 2 microglobulin may be utilized in order to decrease immune response.

          In addition, within a further embodiment of the invention, antisense RNA may be utilized as an anti-tumor agent in order to induce a potent Class I  
20   restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon) due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred  
25   antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

#### 5. Ribozymes

30           Within other aspects of the present invention, gene delivery vehicles are provided which produce ribozymes upon infection of a host cell. Briefly, ribozymes are

used to cleave specific RNAs and are designed such that it can only affect one specific RNA sequence. Generally, the substrate binding sequence of a ribozyme is between 10 and 20 nucleotides long. The length of this sequence is sufficient to allow a hybridization with target RNA and disassociation of the ribozyme from the cleaved  
5 RNA.

A wide variety of ribozymes may be utilized within the context of the present invention, including for example, Group I intron ribozymes (Cech et al., U.S. Patent No. 4,987,071); hairpin ribozymes (Hampel et al., *Nucl. Acids Res.* 18:299-304, 1990, U.S. Patent No. 5,254,678 and European Patent Publication No. 0 360 257),  
10 hammerhead ribozymes (Rossi, J.J. et al., *Pharmac. Ther.* 50:245-254, 1991; Forster and Symons, *Cell* 48:211-220, 1987; Haseloff and Gerlach, *Nature* 328:596-600, 1988; Walbot and Bruening, *Nature* 334:196, 1988; Haseloff and Gerlach, *Nature* 334:585, 1988), hepatitis delta virus ribozymes (Perrotta and Been, *Biochem.* 31:16, 1992); RNase P ribozymes (Takada et al., *Cell* 35:849, 1983); as well as other types of  
15 ribozymes (*see e.g.*, WO 95/29241, and WO 95/31551). Further examples of ribozymes include those described in U.S. Patent Nos. 5,116,742, 5,225,337 and 5,246,921.

#### 6. Proteins and other cellular constituents

Within other aspects of the present invention, a wide variety of proteins  
20 or other cellular constituents may be carried and/or expressed by the gene delivery vehicles provided herein. Representative examples of such proteins include native or altered cellular components, as well as foreign proteins or cellular constituents, found in for example, viruses, bacteria, parasites or fungus.

##### 25 a. Altered Cellular Components

Within one embodiment, gene delivery vehicles are provided which direct the expression of an immunogenic, non-tumorigenic, altered cellular component (*see, e.g.*, WO 93/10814). As utilized herein, the term "immunogenic" refers to altered cellular components which are capable, under the appropriate conditions, of causing an  
30 immune response. This response must be cell-mediated, and may also include a humoral response. The term "non-tumorigenic" refers to altered cellular components

which will not cause cellular transformation or induce tumor formation in nude mice. The phrase "altered cellular component" refers to proteins and other cellular constituents which are either associated with rendering a cell tumorigenic, or are associated with tumorigenic cells in general, but are not required or essential for rendering the cell tumorigenic.

Briefly, before alteration, the cellular components may be essential to normal cell growth and regulation and include, for example, proteins which regulate intracellular protein degradation, transcriptional regulation, cell-cycle control, and cell-cell interaction. After alteration, the cellular components no longer perform their regulatory functions and, hence, the cell may experience uncontrolled growth. Representative examples of altered cellular components include ras<sup>\*</sup>, p53<sup>\*</sup>, Rb<sup>\*</sup>, altered protein encoded by the Wilms' tumor gene, ubiquitin<sup>\*</sup>, mucin<sup>\*</sup>, protein encoded by the DCC, APC, and MCC genes, the breast cancer gene BRCA1<sup>\*</sup>, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor.

Once a sequence encoding the altered cellular component has been obtained, it is necessary to ensure that the sequence encodes a non-tumorigenic protein. Various assays which assess the tumorigenicity of a particular cellular component are known and may easily be accomplished. Representative assays include a rat fibroblast assay, tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

Tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of a particular cellular component. Nude mice lack a functional cellular immune system (*i.e.*, do not possess CTLs), and therefore provide a useful *in vivo* model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if infected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment an alphavirus vector construct is administered to syngeneic murine cells, followed by injection into nude

mice. The mice are visually examined for a period of 2 to 8 weeks after injection in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanella et al., *J. Natl. Cancer Inst.* 48:1531-1533, 1972; Furesz et al., *Abnormal Cells, New Products and Risk*, Hopps and  
5 Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std.* 13:135-141, 1985.)

Tumorigenicity may also be assessed by visualizing colony formation in soft agar (Macpherson and Montagnier, *Virol.* 23:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (*i.e.*, cells will stop proliferating  
10 when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

If the altered cellular component is associated with making the cell tumorigenic, then it is necessary to make the altered cellular component non-  
15 tumorigenic. For example, within one embodiment the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular component. In addition, it is necessary that any truncation leave  
20 intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

25 As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA,  
30 which detects the presence of antibodies against the newly introduced vector, as well as

assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

As noted above, within another aspect of the present invention, several different altered cellular components may be co-expressed in order to form a general anti-cancer therapeutic. Generally, it will be evident to one of ordinary skill in the art that a variety of combinations can be made. Within preferred embodiments, this therapeutic may be targeted to a particular type of cancer. For example, nearly all colon cancers possess mutations in ras, p53, DCC APC or MCC genes. An alphavirus vector construct which co-expresses a number of these altered cellular components may be administered to a patient with colon cancer in order to treat all possible mutations. This methodology may also be utilized to treat other cancers. Thus, an alphavirus vector construct which co-expresses mucin<sup>\*</sup>, ras<sup>\*</sup>, neu, BRCA1<sup>\*</sup> and p53<sup>\*</sup> may be utilized to treat breast cancer.

15                   b.     Antigens from foreign organisms or other pathogens

Within other aspects of the present invention, vectors are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., *E. coli*, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Feline Leukemia Virus ("FeLV"), immunodeficiency viruses such as Feline Immunodeficiency Virus ("FIV") or Human Immunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "HBV" and "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatibility Complex ("MHC") class I presentation, MHC Class II presentation, or both.

Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens (*see, e.g.,* WO 93/15207). Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., *Science* 213:406-411, 1981; Tiollais et al., *Nature* 317:489-495, 1985; and Ganem and Varmus, *Ann. Rev. Biochem.* 56:651-693, 1987; *see also* EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Patent Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAg), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (*see* Blum et al., *TIG* 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

The HBsAg (designated "large," "middle" and "small") are encoded by three regions of the Hepatitis B genome: S, pre-S2 and pre-S1. The large protein, which has a length varying from 389 to 400 amino acids, is encoded by pre-S1, pre-S2, and S regions, and is found in glycosylated and non-glycosylated forms. The middle protein is 281 amino acids long and is encoded by the pre-S2 and S regions. The small protein is 226 amino acids long and is encoded by the S region. It exists in two forms, glycosylated (GP 27<sup>s</sup>) and non-glycosylated (P24<sup>s</sup>). If each of these regions are expressed separately, the pre-S1 region will code for a protein of approximately 119 amino acids, the pre-S2 region will code for a protein of approximately 55 amino acids, and the S region will code for a protein of approximately 226 amino acids.

As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular



combinations of antigens may be preferred for administration in particular geographic regions.

Also presented by HBV are *pol* ("HBV *pol*"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV *pol* may be determined utilizing methods described herein, utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF 5 and ORF 6 (Miller et al., *Hepatology* 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein.

As noted above, at least one immunogenic portion of a hepatitis B antigen is incorporated into a gene delivery vehicle. The immunogenic portion(s) which are incorporated into the gene delivery vehicles may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITES (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA, which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays and proliferation assays.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T cell receptor repertoire recognizes the same antigenic determinants recognized by human T cells. In both systems, the CTL response

generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med.* 173:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992). Particularly preferred immunogenic portions for  
5 incorporation into alphavirus vector constructs include HBeAg, HBcAg and HBsAg. Additional immunogenic portions of the hepatitis B virus may be obtained by truncating the coding sequence at various locations including, for example, the following sites: *Bst* I, *Ssp* I, *Ppu* MI, and *Msp* I (Valenzuela et al., *Nature* 280:815-19, 1979; Valenzuela et al., *Animal Virus Genetics: ICN/UCLA Symp. Mol. Cell Biol.*, 1980, B.  
10 N. Fields and R. Jaenisch (eds.), pp. 57-70, New York: Academic).

As noted above, more than one immunogenic portion may be incorporated into the gene delivery vehicles. For example, a gene delivery vehicle may express (either separately or as one construct) all or immunogenic portions of HBcAg, HBeAg, HBsAg, HBxAg, as well as immunogenic portions of HCV antigens.

15

#### 7. Sources for Heterologous Sequences

Sequences which encode the above-described proteins may be readily obtained from a variety of sources, including for example, depositories such as the American Type Culture Collection (ATCC, Rockville, MD), or from commercial  
20 sources such as British Bio-Technology Limited (Cowley, Oxford, England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids); BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contain sequences encoding alpha interferon), ATCC Nos.  
25 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No 67024 (which contain a sequence which encodes Interleukin-1b); ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2); ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3); ATCC No. 57592 (which contains sequences encoding  
30 Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding

Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Sequences which encode altered cellular components as described above may be readily obtained from a variety of sources. For example, plasmids which  
5 contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, MD), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a G to T mutation in the 12th codon of ras), and  
10 ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (*see*, for example, ATCC No. 41001, which contains a sequence which encodes the normal ras protein; ATCC No. 57103, which encodes abl; and ATCC Nos. 59120 or 59121, which encode the bcr  
15 locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (*sée* Sambrook et al., *supra.*, 15.3 *et seq.*). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

20 Sequences which encode the above-described viral antigens may likewise be obtained from a variety of sources. For example, molecularly cloned genomes which encode the hepatitis B virus may be obtained from sources such as the American Type Culture Collection (ATCC, Rockville, MD). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane  
25 particles) (*see* Figure 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the *Bam* HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981).

Alternatively, cDNA sequences which encode the above-described heterologous sequences may be obtained from cells which express or contain the sequences. Briefly, within one embodiment, mRNA from a cell which expresses the  
30 gene of interest is reverse transcribed with reverse transcriptase using oligonucleotide

dT or random primers. The single stranded cDNA may then be amplified by PCR (see U.S. Patent Nos. 4,683,202; 4,683,195 and 4,800,159. See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double-stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence-specific DNA primers, dATP, dCTP, dGTP and dTTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described proteins may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (e.g., APB DNA synthesizer model 392 (Foster City, CA)).

#### G. Alphavirus Packaging / Producer Cell Lines

Within further aspects of the invention, alphavirus packaging and producer cell lines are provided. In particular, within one aspect of the present invention, alphavirus packaging cell lines are provided wherein the viral structural proteins are supplied in *trans* from one or more stably transformed expression vectors, and are able to encapsidate transfected, transduced, or intracellularly produced vector RNA transcripts in the cytoplasm and release infectious packaged vector particles through the cell membrane. In preferred embodiments, the structural proteins necessary for packaging are synthesized at high levels only after induction by the RNA vector replicon itself or some other provided stimulus, and the transcripts encoding these structural proteins are capable of cytoplasmic amplification in a manner that will allow expression levels sufficient to mimic that of a natural viral infection. Furthermore, in other embodiments, expression of a selectable marker is operably linked to the structural protein expression cassette. Such a linked selectable marker allows efficient generation of functional, stably transformed PCL.

For example, alphavirus RNA vector replicon molecules of the desired phenotype to be packaged, which are themselves capable of autocatalytic replication in the cell cytoplasm, can be introduced into the packaging cells as *in vitro* transcribed

RNA, recombinant alphavirus particles, or as alphavirus cDNA vector constructs. The RNA vector transcripts then replicate to high levels, stimulate amplification of the structural protein gene transcript(s) and subsequent protein expression, and are subsequently packaged by the viral structural proteins, yielding infectious vector particles. The intracellular expression of alphavirus proteins and/or vector RNA above certain levels may result in cytotoxic effects in packaging or producer cell lines. Therefore, within certain embodiments of the invention, it may be desirable for these elements to be derived from virus variants selected for reduced cytotoxicity of their expressed structural proteins, reduced inhibition of host macromolecular synthesis, and/or the ability to establish persistent infection.

To optimize vector packaging cell line performance and final vector titer, successive cycles of gene transfer and vector packaging may be performed. For example, supernatants containing infectious packaged vector particles derived from vector transfection of the packaging cell lines, can be used to infect or "transduce" a fresh monolayer of alphavirus packaging cells. Successive transductions with packaged vector particles and fresh packaging cells may be preferred over nucleic acid transfection because of its higher RNA transfer efficiency into cells, optimized biological placement of the vector in the cell, and ability to "scale-up" the process for vector production from increasingly larger numbers of packaging cells. This leads to higher expression and higher titer of packaged infectious recombinant alphavirus vector.

Within other aspects of the invention, a stably integrated or episomally maintained DNA expression vector can be used to produce the alphavirus vector RNA molecule within the cell. Briefly, such a DNA expression vector can be configured, in preferred embodiments, to be inducible, such that transcription of the alphavirus vector RNA occurs only when cells have been propagated to a desired density, and are subsequently induced. Once transcribed, the alphavirus vector maintains the ability to self-replicate autocatalytically and triggers a cascade of events that culminate in packaged vector particle production. This approach allows for continued vector expression over extended periods of culturing because the integrated DNA vector expression system is maintained through a drug or other selection marker and the DNA

system, once induced, will constitutively express unaltered RNA vector replicons which cannot be diluted out by defective RNA copies. Production of larger-scale, high titer packaged alphavirus vector is possible in this alphavirus "producer cell line" configuration, the DNA-based alphavirus vector is introduced initially into the packaging cell line by transfection, since size restrictions could prevent packaging of the expression vector into a viral vector particle for transduction.

#### H. Pharmaceutical Compositions

As noted above, the present invention also provides pharmaceutical compositions comprising the gene delivery vehicles described herein in combination with a pharmaceutically acceptable carrier, diluent, or recipient. For example, within one embodiment, RNA or DNA vector constructs of the present invention can be lyophilized for long term storage and transport, and may be reconstituted prior to administration using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components which enhance the activity or physically protect the reconstituted nucleic acid preparation. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, lipid formulations, or other components. Lyophilized or dehydrated recombinant vectors may be reconstituted with any convenient volume of water or the reconstituting agents noted above that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

Recombinant alphavirus particles or infectious recombinant virus (both referred to as virus below) may be preserved either in crude or purified forms. In order to produce virus in a crude form, producing cells may first be cultivated in a bioreactor or flat stock culture, wherein viral particles are released from the cells into the culture media. Virus may then be preserved in crude form by first adding a sufficient amount of a formulation buffer to the culture media containing the recombinant virus to form an aqueous suspension. Within certain preferred embodiments, the formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural

additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The recombinant virus can also be preserved in a purified form. More specifically, prior to the addition of the formulation buffer, the crude recombinant virus  
5 described above may be clarified by passing it through a filter and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Nortborough, MA). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated in order to remove excess media components and to establish the recombinant virus in a more desirable buffered solution. The diafiltrate  
10 is then passed over a Sephadex S-500 gel column and a purified recombinant virus is eluted. A sufficient amount of formulation buffer is then added to this eluate in order to reach a desired final concentration of the constituents and to minimally dilute the recombinant virus. The aqueous suspension may then be stored, preferably at  $-70^{\circ}\text{C}$ , or immediately dried. As above, the formulation buffer may be an aqueous solution that  
15 contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

Crude recombinant virus may also be purified by ion exchange column chromatography. Briefly, crude recombinant virus may be clarified by first passing it through a filter, followed by loading the filtrate onto a column containing a highly  
20 sulfonated cellulose matrix. The recombinant virus may then be eluted from the column in purified form by using a high salt buffer, and the high salt buffer exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation buffer is then added, as discussed above, to the purified recombinant virus and the aqueous suspension is either dried immediately or  
25 stored, preferably at  $-70^{\circ}\text{C}$ .

The aqueous suspension in crude or purified form can be dried by lyophilization or evaporation at ambient temperature. Briefly, lyophilization involves the steps of cooling the aqueous suspension below the gas transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water  
30 from the cooled suspension by sublimation to form a lyophilized virus. Within one

embodiment, aliquots of the formulated recombinant virus are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips et al. (*Cryobiology* 18:414, 1981) is used to lyophilize the formulated recombinant virus, preferably from a temperature of -40°C to -45°C. The resulting composition contains less than 10% water by weight of the lyophilized virus. Once lyophilized, the recombinant virus is stable and may be stored at -20°C to 25°C, as discussed in more detail below.

Within the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. Within one embodiment, water is removed through spray-drying (EP 520,748). Within the spray-drying process, the aqueous suspension is delivered into a flow of preheated gas, usually air, whereupon water rapidly evaporates from droplets of the suspension. Spray-drying apparatus are available from a number of manufacturers (e.g., Drytec, Ltd., Tonbridge, England; Lab-Plant, Ltd., Huddersfield, England). Once dehydrated, the recombinant virus is stable and may be stored at -20°C to 25°C. Within the methods described herein, the resulting moisture content of the dried or lyophilized virus may be determined through use of a Karl-Fischer apparatus (EM Science Aquastar' VIB volumetric titrator, Cherry Hill, NJ), or through a gravimetric method.

The aqueous solutions used for formulation, as previously described, are preferably composed of a saccharide, high molecular weight structural additive, a buffering component, and water. The solution may also include one or more amino acids. The combination of these components act to preserve the activity of the recombinant virus upon freezing and lyophilization or drying through evaporation. Although one saccharide that can be utilized is lactose, other saccharides may likewise be utilized including, for example, sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose or galactose. In addition, combinations of saccharides can be used, for example, lactose and mannitol, or sucrose and mannitol. A particularly preferred concentration of lactose is 3%-4% by weight. Preferably, the concentration of the saccharide ranges from 1% to 12% by weight.



The high molecular weight structural additive aids in preventing viral aggregation during freezing and provides structural support in the lyophilized or dried state. Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 m.w. A preferred high molecular weight structural additive is human serum albumin. However, other substances may also be used, such as hydroxyethyl-cellulose, hydroxymethyl-cellulose, dextran, cellulose, gelatin, or povidone. A particularly preferred concentration of human serum albumin is 0.1% by weight. Preferably, the concentration of the high molecular weight structural additive ranges from 0.1% to 10% by weight.

10 The amino acids, if present, function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, amino acids function to further preserve viral infectivity during sublimation of the cooled aqueous suspension and while in the lyophilized state. A preferred amino acid is arginine, but other amino acids such as lysine, ornithine, serine, glycine, glutamine, asparagine, glutamic acid or  
15 aspartic acid can also be used. A particularly preferred arginine concentration is 0.1% by weight. Preferably, the amino acid concentration ranges from 0.1% to 10% by weight.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers may be used, depending on the pH range  
20 desired, preferably between 7.0 and 7.8. Suitable buffers include phosphate buffer and citrate buffer. A particularly preferred pH of the recombinant virus formulation is 7.4, and a preferred buffer is tromethamine.

In addition, it is preferable that the aqueous solution contain a neutral salt which is used to adjust the final formulated recombinant alphavirus to an  
25 appropriate iso-osmotic salt concentration. Suitable neutral salts include sodium chloride, potassium chloride or magnesium chloride. A preferred salt is sodium chloride.

Aqueous solutions containing the desired concentration of the components described above may be prepared as concentrated stock solutions.

It will be evident to those skilled in the art, given the disclosure provided herein, that it may be preferable to utilize certain saccharides within the aqueous solution when the lyophilized virus is intended for storage at room temperature. More specifically, it is preferable to utilize disaccharides, such as lactose or trehalose, particularly for storage at room temperature.

The lyophilized or dehydrated viruses of the subject invention may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the reconstituted virus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted virus. Lyophilized or dehydrated recombinant virus may be reconstituted with any convenient volume of water or the reconstituting agents noted above that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

#### I. Methods for Utilizing Gene Delivery Vehicles

As noted above, the present invention also provides methods for delivering a selected heterologous sequence to a vertebrate (*e.g.*, a mammal such as a human or other warm-blooded animal such as a horse, cow, pig, sheep, dog, cat, rat or mouse) or insect, comprising the step of administering to a vertebrate or insect a gene delivery vehicle as described herein which is capable of expressing the selected heterologous sequence. Such gene delivery vehicles may be administered either directly (*e.g.*, intravenously, intramuscularly, intraperitoneally, subcutaneously, orally, rectally, intraocularly, intranasally), or by various physical methods such as lipofection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Fung et al., *Proc. Natl. Acad. Sci. USA* 80:353-357, 1983; Seeger et al., *Proc. Natl. Acad. Sci. USA* 81:5849-5852; Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991); liposomes of several types (*see, e.g.*, Wang et al., *PNAS* 84:7851-7855, 1987);  $\text{CaPO}_4$  (Dubensky et al., *PNAS* 81:7529-7533, 1984); DNA ligand (Wu et al., *J. Biol. Chem.* 264:16985-

16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3:147-154, 1992); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. In addition, the gene  
5 delivery vehicles may either be administered directly (*i.e.*, *in vivo*), or to cells which have been removed (*ex vivo*), and subsequently returned.

As discussed in more detail below, gene delivery vehicles may be administered to a vertebrate or insect for a wide variety of therapeutic and/or other productive purposes, including for example, for the purpose of stimulating a specific  
10 immune response; inhibiting the interaction of an agent with a host cell receptor; to express a toxic palliative, including for example, conditional toxic palliatives; to immunologically regulate the immune system; to prevent cell division, to express markers, for replacement gene therapy, to promote wound healing and/or to produce a recombinant protein. These and other uses are discussed in more detail below.

15

#### 1. Immunostimulation

Within one aspect of the present invention, compositions and methods are provided for administering a gene delivery vehicle which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune  
20 diseases. Representative examples of such diseases include viral infections such as HIV, HBV, HCV, HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease. More specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a pathogenic agent, such that  
25 the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents include bacteria, fungi, parasites, viruses and cancer cells.

Within one embodiment of the invention the pathogenic agent is a virus, and methods are provided for stimulating a specific immune response and inhibiting viral spread by using a gene delivery vehicle that directs the expression of an antigen or  
30 modified form thereof to susceptible target cells capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying

cellular receptors required for viral interactions. Expression of the vector nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the gene delivery vehicle is preferably designed to express a modified form of the antigen which will stimulate an immune response and  
5 which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogues thereof (*e.g.*, Altmann et al., *Nature* 338:512, 1989). Cells infected with gene delivery vehicles are expected to do this efficiently because  
10 they closely mimic genuine viral infection and because they: (a) are able to infect non-replicating cells, (b) do not integrate into the host cell genome, (c) are not associated with any life threatening diseases, and (d) express high levels of heterologous protein. Because of these differences, gene delivery vehicles can easily be thought of as safe viral vectors which can be used on healthy individuals for vaccine use.

15 This aspect of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and low levels of viral antigens, relative to heterologous genes, are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the  
20 recombinant alphavirus, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against  
25 antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response may also be achieved by transferring to an  
30 appropriate immune cell (such as a T lymphocyte) the gene for the specific T cell

receptor which recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), for an immunoglobulin which recognizes the antigen of interest, or for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus, the gene delivery vehicle cells may be used as an immunostimulant,  
5 immunomodulator, or vaccine.

In another embodiment of the invention, methods are provided for producing inhibitor palliatives wherein gene delivery vehicles deliver and express defective interfering viral structural proteins, which inhibit viral assembly. Such gene delivery vehicles may encode defective *gag*, *pol*, *env* or other viral particle proteins or  
10 peptides and these would inhibit in a dominant fashion the assembly of viral particles. This occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

In another embodiment of the invention, methods are provided for the expression of inhibiting peptides or proteins specific for viral protease. Briefly, viral  
15 protease cleaves the viral *gag* and *gag/pol* proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. As an example, the HIV protease is known to be an aspartyl protease and these are known to be inhibited by peptides made from amino acids from protein or analogues. Gene delivery vehicles to inhibit HIV will express one  
20 or multiple fused copies of such peptide inhibitors.

Another embodiment involves the delivery of suppressor genes which, when deleted, mutated, or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a gene delivery vehicle leads to regression of the tumor phenotype in these cells. Examples of such cancers are  
25 retinoblastoma and Wilms Tumor. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the alphavirus vector delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

In yet another embodiment, the gene delivery vehicle provides a  
30 therapeutic effect by transcribing a ribozyme (an RNA enzyme) (Haseloff and Gerlach,

*Nature* 334:585, 1989) which will cleave and hence inactivate RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA species such as a RNA or a retroviral genome. Additional specificity may be achieved in some cases by making this a conditional toxic palliative (*see below*).

One way of increasing the effectiveness of inhibitory palliatives is to express viral inhibitory genes in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, gene delivery vehicles may be delivered which inhibit HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

## 2. Blocking Agents

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors produced by themselves or other cells. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers or other proliferative conditions (*e.g.*, restenosis), cells may respond inappropriately or not at all to signals from other cells or factors, or specific factors may be mutated, overexpressed, or underexpressed, resulting in loss of appropriate cell cycle control. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by producing, *in vivo*, an analogue to either of the partners in an interaction. Alternatively, cell cycle control may be restored by preventing the transition from one phase to another (*e.g.*, G1 to S phase) using a blocking factor which is absent or underexpressed. This blocking action may occur intracellularly, on the cell membrane, or extracellularly, and the action of an alphavirus

vector carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

In the case of HIV, the two agents of interaction are the gp 120/gp 41  
5 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a gene delivery vehicle expressing either an HIV *env* analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp  
10 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Administration of a gene delivery vehicle encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule. Efficacy of treatment can be assayed by measuring the usual indicators of disease progression, including antibody  
15 level, viral antigen production, infectious HIV levels, or levels of nonspecific infections.

In the case of uncontrolled proliferative states, such as cancer or restenosis, cell cycle progression may be halted by the expression of a number of different factors that affect signaling by cyclins or cyclin-dependent kinases (CDK).  
20 For example, the cyclin-dependent kinase inhibitors, p16, p21, and p27 each regulate cyclin:CDK mediated cell cycle signaling. Overexpression of these factors within a cell by a gene delivery vehicle results in a cytostatic suppression of cell proliferation. Other factors that may be used therapeutically, as blocking agents or targets to disrupt cell proliferation, include, for example, wild-type or mutant Rb, p53, Myc, Fos, Jun, PCNA,  
25 GAX, lentiviral *vpr* and p15. Within related embodiments, cardiovascular diseases such as restenosis or atherosclerosis may be treated or prevented with vectors that express products which promote re-endothelialization, or vascular remodeling (e.g., VEGF, TFPI, SOD).

### 3. Expression of Palliatives

Techniques similar to those described above can be used to produce gene delivery vehicles which direct the expression of an agent (or "palliative") which is capable of inhibiting a function of a pathogenic agent or gene. Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for a cancerous cell, cancer-promoting growth factor, or uncontrolled proliferative condition (e.g., restenosis) include viability, cell replication, altered susceptibility to external signals (e.g., contact inhibition), and lack of production or production of mutated forms of anti-oncogene proteins.

#### 15 a. Inhibitor Palliatives

In one aspect of the present invention, the gene delivery vehicle directs the expression of a gene which can interfere with a function of a pathogenic agent, for instance in viral or malignant diseases. Such expression may either be essentially continuous or in response to the presence in the cell of another agent associated either with the pathogenic condition or with a specific cell type (an "identifying agent"). In addition, vector delivery may be controlled by targeting vector entry specifically to the desired cell type (for instance, a virally infected or malignant cell) as discussed above.

One method of administration is leukaphoresis, in which about 20% of an individual's PBLs are removed at any one time and manipulated *in vitro*. Thus, approximately  $2 \times 10^9$  cells may be treated and replaced. Repeat treatments may also be performed. Alternatively, bone marrow may be treated and allowed to amplify the effect as described above. In addition, packaging cell lines producing a vector may be directly injected into a subject, allowing continuous production of recombinant virions.

In one embodiment, gene delivery vehicles which express RNA complementary to key pathogenic gene transcripts (for example, a viral gene product or an activated cellular oncogene) can be used to inhibit translation of that transcript into



protein, such as the inhibition of translation of the HIV tat protein. Since expression of this protein is essential for viral replication, cells containing the gene delivery vehicle would be resistant to HIV replication.

In a second embodiment, where the pathogenic agent is a single-stranded virus having a packaging signal, RNA complementary to the viral packaging signal (e.g., an HIV packaging signal when the palliative is directed against HIV) is expressed, so that the association of these molecules with the viral packaging signal will, in the case of retroviruses, inhibit stem loop formation or tRNA primer binding required for proper encapsidation or replication of the alphavirus RNA genome.

10 In a third embodiment, a gene delivery vehicle may be introduced which expresses a palliative capable of selectively inhibiting the expression of a pathogenic gene, or a palliative capable of inhibiting the activity of a protein produced by the pathogenic agent. In the case of HIV, one example is a mutant tat protein which lacks the ability to transactivate expression from the HIV LTR and interferes (in a  
15 transdominant manner) with the normal functioning of tat protein. Such a mutant has been identified for HTLV II tat protein ("XII Leu'" mutant; see Wachsman et al., *Science* 235:674, 1987). A mutant transrepressor tat should inhibit replication much as has been shown for an analogous mutant repressor in HSV-1 (Friedmann et al., *Nature* 335:452, 1988).

20 Such a transcriptional repressor protein can be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are  
25 introduced to the system, a mutant with the appropriate properties (i.e., represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector/tat system may be used to assure that surviving cell clones are not caused by endogenous mutations in these genes.  
30 A battery of randomly mutagenized tat genes are then introduced into these cells using a

"rescuable" alphavirus vector (*i.e.*, one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This  
5 procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

b. Conditional Toxic Palliatives

Another approach for inhibiting a pathogenic agent is to express a  
10 palliative which is toxic for the cell expressing the pathogenic condition. In this case, expression of the palliative from the gene delivery vehicle should be limited by the presence of an entity associated with the pathogenic agent, such as a specific viral RNA sequence identifying the pathogenic state, in order to avoid destruction of nonpathogenic cells.

15 In one embodiment of this method, a gene delivery vehicle can be utilized to express a toxic gene (as discussed above) from a cell-specific responsive vector. In this manner, rapidly replicating cells, which contain the RNA sequences capable of activating the cell-specific responsive vectors, are preferentially destroyed by the cytotoxic agent produced by the gene delivery vehicle.

20 In a similar manner to the preceding embodiment, the gene delivery vehicle can carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. This gene may have no equivalent in mammalian cells and might come from organisms such as a virus, bacterium, fungus, or protozoan. An example of this would be the *E. coli* guanine  
25 phosphoribosyl transferase gene product, which is lethal in the presence of thioxanthine (*see* Besnard et al., *Mol. Cell. Biol.* 7:4139-4141, 1987). Conditionally lethal gene products of this type (also referred to as "prodrugs converting enzymes" above) have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become  
30 effective cytotoxic agents. The conditionally lethal gene product could also metabolize

a nontoxic drug which is not a purine or pyrimidine analogue to a cytotoxic form (see Searle et al., *Brit. J. Cancer* 53:377-384, 1986).

Mammalian viruses in general tend to have "immediate early" genes which are necessary for subsequent transcriptional activation from other viral promoter elements. RNA sequences of this nature are excellent candidates for activating alphavirus vectors intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes expressed from alphavirus cell-specific vectors responsive to these viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human and interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like HSVTK, for example, in response to interferon production could result in the destruction of cells infected with a variety of different viruses.

In another aspect of the present invention, gene delivery vehicles are provided which direct the expression of a gene product capable of activating an otherwise inactive precursor into an active inhibitor of the pathogenic agent. For example, the HSVTK gene product may be used to more effectively metabolize potentially antiviral nucleoside analogues such as AZT or ddC. The HSVTK gene may be expressed under the control of a cell-specific responsive vector and introduced into these cell types. AZT (and other nucleoside antivirals) must be metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus, HIV replication (Furmam et al., *Proc. Natl. Acad. Sci. USA* 83:8333-8337, 1986). Constitutive expression of HSVTK (a nucleoside and nucleoside kinase with very broad substrate specificity) results in more effective metabolism of these drugs to their biologically active nucleotide triphosphate form. AZT or ddC therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular

nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

Administration of these gene delivery vehicles to human T cell and macrophage/monocyte cell lines can increase their resistance to HIV in the presence of  
5 AZT and ddC compared to the same cells without retroviral vector treatment. Treatment with AZT would be at lower than normal levels to avoid toxic side effects but still efficiently inhibit the spread of HIV. The course of treatment would be as described for the blocker.

In one embodiment, the gene delivery vehicle carries a gene specifying a  
10 product which is not in itself toxic but, when processed or modified by a protein such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the gene delivery vehicle could carry a gene encoding a proprotein for ricin A chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxin ricin or diphtheria A chains could be  
15 cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

In another embodiment, the gene delivery vehicle may express a "reporting product" on the surface of the target cells in response to the presence of an identifying agent in the cells (such as expression of a viral gene). This surface protein  
20 can be recognized by a cytotoxic agent, such as antibodies for the reporting protein, or by cytotoxic T cells. In a similar manner, such a system can be used as a detection system (see below) to simply identify those cells having a particular gene which expresses an identifying protein.

Similarly, in another embodiment, a surface protein could be expressed  
25 which would itself be therapeutically beneficial. In the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV *env* intracellularly could inhibit the formation of viable viral particles, much as soluble CD4 has been shown to do for free  
30 virus, but without the problem of systematic clearance and possible immunogenicity,

since the protein will remain membrane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV *env* complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-*env* present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

10 In another embodiment, the gene delivery vehicle can provide a ribozyme which will cleave and inactivate RNA molecules essential for viability of the vector infected cell. By making ribozyme production dependent on a specific RNA sequence corresponding to the pathogenic state, such as HIV *tat*, toxicity is specific to the pathogenic state.

15

#### 4. Expression of Markers

The above-described technique of expressing a palliative in a cell in response to a specific RNA sequence can also be modified to enable detection of a particular gene in a cell which expresses an identifying protein (for example, a gene carried by a particular virus), and hence enable detection of cells carrying that virus. In addition, this technique enables the detection of viruses (such as HIV) in a clinical sample of cells carrying an identifying protein associated with the virus.

This modification can be accomplished by providing a genome coding for a product, the presence of which can be readily identified (the "marker product"), in a gene delivery vehicle which responds to the presence of the identifying protein in the infected cells. For example, HIV, when it infects suitable cells, makes *tat* and *rev*. The indicator cells can thus be provided with a genome (such as by infection with an appropriate recombinant alphavirus) which codes for a marker gene, such as the alkaline phosphatase gene,  $\beta$ -galactosidase gene, or the luciferase gene which is expressed by the recombinant alphavirus upon activation by the *tat* and/or *rev* RNA transcript. In the case of  $\beta$ -galactosidase or alkaline phosphatase, exposing the cells to substrate

analogues results in a color or fluorescence change if the sample is positive for HIV. In the case of luciferase, exposing the sample to luciferin will result in luminescence if the sample is positive for HIV. For intracellular enzymes such as  $\beta$ -galactosidase, the viral titre can be measured directly by counting colored or fluorescent cells, or by making  
5 cell extracts and performing a suitable assay. For the membrane bound form of alkaline phosphatase, virus titre can also be measured by performing enzyme assays on the cell surface using a fluorescent substrate. For secreted enzymes, such as an engineered form of alkaline phosphatase, small samples of culture supernatant are assayed for activity, allowing continuous monitoring of a single culture over time. Thus, different forms of  
10 this marker system can be used for different purposes. These include counting active virus, or sensitively and simply measuring viral spread in a culture and the inhibition of this spread by various drugs.

Further specificity can be incorporated into the preceding system by testing for the presence of the virus either with or without neutralizing antibodies to that  
15 virus. For example, in one portion of the clinical sample being tested, neutralizing antibodies to HIV may be present; whereas in another portion there would be no neutralizing antibodies. If the tests were negative in the system where there were antibodies and positive where there were no antibodies, this would assist in confirming the presence of HIV.

20 Within an analogous system for an *in vitro* assay, the presence of a particular gene, such as a viral gene, may be determined in a cell sample. In this case, the cells of the sample are infected with a suitable gene delivery vehicle which carries the reporter gene which is only expressed in the presence of the appropriate viral RNA transcript. The reporter gene, after entering the sample cells, will express its reporting  
25 product (such as  $\beta$ -galactosidase or luciferase) only if the host cell expresses the appropriate viral proteins.

These assays are more rapid and sensitive, since the reporter gene can express a greater amount of reporting product than identifying agent present, which results in an amplification effect.

## 5. Immune Down-Regulation

As described above, the present invention also provides gene delivery vehicles capable of suppressing one or more elements of the immune system in target cells infected with the alphavirus. Briefly, specific down-regulation of inappropriate or unwanted immune responses, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products which suppress surface expression of transplantation (MHC) antigen. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells, and prevents terminal glycosylation and translocation of class I MHC to the cell surface. For example, prior to bone marrow transplantation, donor bone marrow cells may be infected with a gp 19-encoding gene delivery vehicle which, upon expression of the gp 19, inhibit the surface expression of MHC class I transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the transplant patient. This may allow an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

An alternative method involves the use of anti-sense message, ribozyme, or other specific gene expression inhibitor specific for T cell clones which are autoreactive in nature. These block the expression of the T cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using the viral vector delivery system.

## 6. Replacement or Augmentation Gene Therapy

One further aspect of the present invention relates to transforming cells of a vertebrate or insect with a gene delivery vehicle which supplies genetic sequences capable of expressing a therapeutic protein. Within one embodiment of the present invention, the gene delivery vehicle is designed to express a therapeutic protein capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic

defect in metabolism, immune regulation, hormonal regulation, enzymatic or membrane associated structural function. This embodiment also describes the gene delivery vehicle capable of transducing individual cells, whereby the therapeutic protein is able to be expressed systemically or locally from a specific cell or tissue, whereby the therapeutic protein is capable of (a) the replacement of an absent or defective cellular protein or enzyme, or (b) supplement production of a defective or low expressed cellular protein or enzyme. Such diseases may include cystic fibrosis, Parkinson's disease, hypercholesterolemia, adenosine deaminase deficiency,  $\beta$ -globin disorders, Hemophilia A & B, Gaucher's disease, diabetes and leukemia.

10 As an example of the present invention, a gene delivery vehicle can be constructed and utilized to treat Gaucher disease. Briefly, Gaucher disease is a genetic disorder that is characterized by the deficiency of the enzyme glucocerebrosidase. This type of therapy is an example of a single gene replacement therapy by providing a functional cellular enzyme. This enzyme deficiency leads to the accumulation of  
15 glucocerebroside in the lysosomes of all cells in the body. However, the disease phenotype is manifested only in the macrophages, except in the very rare neuronopathic forms of the disease. The disease usually leads to enlargement of the liver and spleen and lesions in the bones. (For a review, see *Science* 256:794, 1992, and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver et al., vol. 2, p. 1677).

20 Gene delivery vehicles can similarly be utilized to deliver a wide variety of therapeutic proteins in order to treat, cure, prevent a disease or disease process. Representative examples of such genes include, but are not limited to, insulin (see U.S. 4,431,740 and BE 885196A), hemoglobin (Lawn et al., *Cell* 21:647-51, 1980), erythropoietin (EPO; see U.S. 4,703,008), megakaryocyte growth and differentiation  
25 factor (MGDF), stem cell factor (SCF), G-CSF (Nagata et al., *Nature* 319:415-418, 1986), GM-CSF, M-CSF (see WO 8706954), the flt3 ligand (Lyman et al. (1993), *Cell* 75:1157-1167), EGF, acidic and basic FGF, PDGF, members of the interleukin or interferon families, *supra*, neurotropic factors (e.g., BDNF; Rosenthal et al., *Endocrinology* 129:1289-1294, 1991, NT-3; see WO 9103569, CNTF; see WO  
30 9104316, NGF; see WO 9310150), coagulation factors (e.g., factors VIII and IX),



thrombolytic factors such as t-PA (*see* EP 292009, AU 8653302 and EP 174835) and streptokinase (*see* EP 407942), human growth hormone (*see* JP 94030582 and U.S. 4,745,069) and other animal somatotropins, integrins and other cell adhesion molecules, such as ICAM-1 and ELAM (*see also* other "heterologous sequences" discussed above),  
5 and other growth factors, such as IGF-I and IGF-II, TGF- $\beta$ , osteogenic protein-1 (Ozkaynak et al., *EMBO J.* 9:2085-2093, 1990), and other bone morphogenetic proteins (*e.g.*, BMP-4, Nakase et al, *J. Bone Miner. Res.* 9:651-659, 1994).

#### 7. Lymphokines and Lymphokine Receptors

10 As noted above, the present invention also provides gene delivery vehicles which can, among other functions, direct the expression of one or more cytokines or cytokine receptors. Briefly, in addition to their role as cancer therapeutics, cytokines can have negative effects resulting in certain pathological conditions. For example, most resting T-cells, B cells, large granular lymphocytes and monocytes do  
15 not express IL-2R (receptor). In contrast to the lack of IL-2R expression on normal resting cells, IL-2R is expressed by abnormal cells in patients with certain leukemias (ATL, Hairy-cell, Hodgkins, acute and chronic granulocytic), autoimmune diseases, and is associated with allograft rejection. Interestingly, in most of these patients the serum concentration of a soluble form of IL-2R is elevated. Therefore, with certain  
20 embodiments of the invention therapy may be effected by increasing the serum concentration of the soluble form of the cytokine receptor. For example, in the case of IL-2R, a gene delivery vehicle can be engineered to produce both soluble IL-2R and IL-2R, creating a high affinity soluble receptor. In this configuration, serum IL-2 levels would decrease, inhibiting the paracrine loop. This same strategy also may be effective  
25 against autoimmune diseases. In particular, because some autoimmune diseases (*e.g.*, Rheumatoid arthritis, SLE) also are associated with abnormal expression of IL-2, blocking the action of IL-2 by increasing the serum level of receptor may also be utilized in order to treat such autoimmune diseases.

In other cases inhibiting the levels of IL-1 may be beneficial. Briefly,  
30 IL-1 consists of two polypeptides, IL-1 and IL-1, each of which has pleiotropic effects. IL-1 is primarily synthesized by mononuclear phagocytes, in response to stimulation by

microbial products or inflammation. There is a naturally occurring antagonist of the IL-1R, referred to as the IL-1 Receptor antagonist ("IL-1Ra"). This IL-1R antagonist has the same molecular size as mature IL-1 and is structurally related to it. However, binding of IL-1Ra to the IL-1R does not initiate any receptor signaling. Thus, this molecule has a different mechanism of action than a soluble receptor, which complexes with the cytokine and thus prevents interaction with the receptor. IL-1 does not seem to play an important role in normal homeostasis. In animals, antibodies to IL-1 receptors reduce inflammation and anorexia due to endotoxins and other inflammation inducing agents.

10 In the case of septic shock, IL-1 induces secondary compounds which are potent vasodilators. In animals, exogenously supplied IL-1 decreases mean arterial pressure and induces leukopenia. Neutralizing antibody to IL-1 reduced endotoxin-induced fever in animals. In a study of patients with septic shock who were treated with a constant infusion of IL-1R for three days, the 28 day mortality was 16% compared to 15 44% in patients who received placebo infusions. In the case of autoimmune disease, reducing the activity of IL-1 reduces inflammation. Similarly, blocking the activity of IL-1 with recombinant receptors can result in increased allograft survival in animals, again presumably by decreasing inflammation.

These diseases provide further examples where gene delivery vehicles 20 may be engineered to produce a soluble receptor or more specifically the IL-1Ra molecule. For example, in patients undergoing septic shock, a single injection of IL-1Ra producing vector particles could replace the current approach requiring a constant infusion of recombinant IL-1R.

Cytokine responses, or more specifically, incorrect cytokine responses 25 may also be involved in the failure to control or resolve infectious diseases. Perhaps the best studied example is non-healing forms of leishmaniasis in mice and humans which have strong, but counterproductive  $T_H2$ -dominated responses. Similarly, lepromatous leprosy is associated with a dominant, but inappropriate  $T_H2$  response. In these conditions, gene delivery vehicles may be useful for increasing circulating 30 levels of IFN gamma, as opposed to the site-directed approach proposed for solid tumor

therapy. IFN gamma is produced by  $T_H$ -1 T-cells, and functions as a negative regulator of  $T_H$ -2 subtype proliferation. IFN gamma also antagonizes many of the IL-4 mediated effects on B-cells, including isotype switching to IgE.

IgE, mast cells and eosinophils are involved in mediating allergic  
5 reaction. IL-4 acts on differentiating T-cells to stimulate  $T_H$ -2 development, while inhibiting  $T_H$ -1 responses. Thus, alphavirus-based gene therapy may also be accomplished in conjunction with traditional allergy therapeutics. One possibility is to deliver a gene delivery vehicle which produces IL4R with small amounts of the offending allergen (*i.e.*, traditional allergy shots). Soluble IL-4R would prevent the  
10 activity of IL-4, and thus prevent the induction of a strong  $T_H$ -2 response.

#### 8. Suicide Vectors

One further aspect of the present invention relates to the use of gene delivery vehicle suicide vectors to limit the spread of wild-type alphavirus in the  
15 packaging/producer cell lines. Briefly, within one embodiment the gene delivery vehicle is comprised of an antisense or ribozyme sequence specific for the wild-type alphavirus sequence generated from an RNA recombination event between the 3' sequences of the junction region of the vector, and the 5' alphavirus structural sequences of the packaging cell line expression vector. The antisense or ribozyme molecule would  
20 only be thermostable in the presence of the specific recombination sequence and would not have any other effect in the alphavirus packaging/producer cell line. Alternatively, a toxic molecule (such as those disclosed herein), may also be expressed in the context of a vector that would only express in the presence of wild-type alphavirus.

#### 25 9. Gene Delivery Vehicles to Prevent the Spread of Metastatic Tumors

One further aspect of the present invention relates to the use of gene delivery vehicles for inhibiting or reducing the invasiveness of malignant neoplasms. Briefly, the extent of malignancy typically relates to vascularization of the tumor. One cause for tumor vascularization is the production of soluble tumor angiogenesis factors  
30 (TAF) (Paweletz et al., *Crit. Rev. Oncol. Hematol.* 9:197, 1989) expressed by some tumors. Within one aspect of the present invention, tumor vascularization may be

slowed utilizing gene delivery vehicles to express antisense or ribozyme RNA molecules specific for TAF. Alternatively, anti-angiogenesis factors (Moses et al., *Science* 248:1408, 1990; Shapiro et al., *PNAS* 84:2238, 1987) may be expressed either alone or in combination with the above-described ribozymes or antisense sequences in order to slow or inhibit tumor vascularization. Alternatively, gene delivery vehicles can also be used to express an antibody specific for the TAF receptors on surrounding tissues.

#### 10. Administration of Gene Delivery Vehicles

Within other aspects of the present invention, methods are provided for administering a gene delivery vehicle to a vertebrate or insect. Briefly, the final mode of gene delivery vehicle administration usually relies on the specific therapeutic application, the best mode of increasing vector potency, and the most convenient route of administration. Generally, this embodiment includes gene delivery vehicles which can be designed to be delivered by, for example, (1) direct injection into the blood stream; (2) direct injection into a specific tissue or tumor; (3) oral administration; (4) nasal inhalation; (5) direct application to mucosal tissues; or (6) *ex vivo* administration of transduced autologous cells into the vertebrate or insect. Within certain embodiments of the invention, for *ex vivo* applications cells can be first removed from a host, positively and/or negatively selected in order to yield a population of cells which is at least partially purified (*e.g.*, CD34<sup>+</sup> stem cells, T cells, or the like), transduced, transfected, or, infected with one of the gene delivery vehicles of the present invention, and reintroduced into either the same host or another individual.

Thus, the therapeutic gene delivery vehicle can be administered in such a fashion such that the vector can (a) transduce a normal healthy cell and transform the cell into a producer of a therapeutic protein or agent which is secreted systemically or locally, (b) transform an abnormal or defective cell, transforming the cell into a normal functioning phenotype, (c) transform an abnormal cell so that it is destroyed, and/or (d) transduce cells to manipulate the immune response.

### 11. Modulation of Transcription Factor Activity

In yet another embodiment, gene delivery vehicles may be utilized in order to regulate the growth control activity of transcription factors in the infected cell. Briefly, transcription factors directly influence the pattern of gene expression through sequence-specific *trans*-activation or repression (Karin, *New Biologist* 21:126-131, 1990). Thus, it is not surprising that mutated transcription factors represent a family of oncogenes. Gene delivery vehicles can be used, for example, to return control to tumor cells whose unregulated growth is activated by oncogenic transcription factors, and proteins which promote or inhibit the binding cooperatively in the formation of homo- and heterodimer *trans*-activating or repressing transcription factor complexes.

One method for reversing cell proliferation would be to inhibit the *trans*-activating potential of the *c-myc*/Max heterodimer transcription factor complex. Briefly, the nuclear oncogene *c-myc* is expressed by proliferating cells and can be activated by several distinct mechanisms, including retroviral insertion, amplification, and chromosomal translocation. The Max protein is expressed in quiescent cells and, independently of *c-myc*, either alone or in conjunction with an unidentified factor, functions to repress expression of the same genes activated by the *myc*/Max heterodimer (Cole, *Cell* 65:715-716, 1991).

Inhibition of *c-myc* or *c-myc*/Max proliferation of tumor cells may be accomplished by the overexpression of Max in target cells controlled by gene delivery vehicles. The Max protein is only 160 amino acids (corresponding to 480 nucleotide RNA length) and is easily incorporated into a gene delivery vehicle either independently, or in combination with other genes and/or antisense/ribozyme moieties targeted to factors which release growth control of the cell.

Modulation of homo/hetero-complex association is another approach to control transcription factor activated gene expression. For example, transport from the cytoplasm to the nucleus of the *trans*-activating transcription factor NF-B is prevented while in a heterodimer complex with the inhibitor protein IB. Upon induction by a variety of agents, including certain cytokines, IB becomes phosphorylated and NF-B is released and transported to the nucleus, where it can exert its sequence-specific *trans*-activating function (Baeuerle and Baltimore, *Science* 242:540-546, 1988). The

dissociation of the NF-B/IB complex can be prevented by masking with an antibody the phosphorylation site of IB. This approach would effectively inhibit the *trans*-activation activity of the NF-IB transcription factor by preventing its transport to the nucleus. Expression of the IB phosphorylation site specific antibody or protein in target cells  
5 may be accomplished with an alphavirus gene transfer vector. An approach similar to the one described here could be used to prevent the formation of the *trans*-activating transcription heterodimer factor AP-1 (Turner and Tijan, *Science* 243:1689-1694, 1989), by inhibiting the association between the *jun* and *fos* proteins.

10           12.    Production of Recombinant Proteins

In another aspect of the present invention, togavirus (including alphavirus) gene delivery vehicles can be utilized to direct the expression of one or more recombinant proteins in eukaryotic cells (*ex vivo*, *in vivo*, or established cell lines). As used herein, a "recombinant protein" refers to a protein, polypeptide, enzyme, or  
15 fragment thereof. Using this approach, proteins having therapeutic or other commercial application can be more cost-effectively produced. Furthermore, proteins produced in eukaryotic cells may be more authentically modified post-translationally (e.g., glycosylated, sulfated, acetylated, etc.), as compared to proteins produced in prokaryotic cells. In addition, such systems may be employed in the *in vivo* production of various  
20 chemical compounds, e.g., fine or specialty chemicals.

Within this aspect, the gene delivery vehicle encoding the desired protein, enzyme, or enzymatic pathway (as may be required for the production of a desired chemical) is transformed, transfected, transduced or otherwise introduced into a suitable eukaryotic cell. Representative examples of proteins which can be produced  
25 using such a system include, but are not limited to, insulin (see U.S. 4,431,740 and BE 885196A), hemoglobin (Lawn et al., *Cell* 21:647-51, 1980), erythropoietin (EPO; see U.S. 4,703,008), megakaryocyte growth and differentiation factor (MGDF), stem cell factor (SCF), G-CSF (Nagata et al., *Nature* 319:415-418, 1986), GM-CSF, M-CSF (see WO 8706954), the flt3 ligand (Lyman et al. (1993), *Cell* 75:1157-1167), EGF, acidic  
30 and basic FGF, PDGF, members of the interleukin or interferon families, *supra*, neurotropic factors (e.g., BDNF; Rosenthal et al., *Endocrinology* 129:1289-1294, 1991,

NT-3; *see* WO 9103569, CNTF; *see* WO 9104316, NGF; *see* WO 9310150), coagulation factors (*e.g.*, factors VIII and IX), thrombolytic factors such as t-PA (*see* EP 292009, AU 8653302 and EP 174835) and streptokinase (*see* EP 407942), human growth hormone (*see* JP 94030582 and U.S. 4,745,069) and other animal  
5 somatotropins, integrins and other cell adhesion molecules, such as ICAM-1 and ELAM (*see also* other "heterologous sequences" discussed above), and other growth factors, such as IGF-I and IGF-II, TGF- $\beta$ , osteogenic protein-1 (Ozkaynak et al., *EMBO J.* 9:2085-2093, 1990), and other bone morphogenetic proteins (*e.g.*, BMP-4, Nakase et al, *J. Bone Miner. Res.* 9:651-659, 1994). As those in the art will appreciate, once  
10 characterized, any gene can be readily cloned into gene delivery vehicles according to the present invention, followed by introduction into a suitable host cell and expression of the desired gene.

Methods for producing recombinant proteins using the vectors and alphavirus packaging cell lines described herein are provided (*see* examples 6 and 7).  
15 Briefly, gene delivery vehicles, in the form of *in vitro* transcribed RNA, plasmid DNA, or recombinant vector particles, which encode recombinant proteins, may be introduced (via transfection or infection) into alphavirus packaging cell lines (PCLs) such that only a small fraction of the cultured cells ( $\leq 1\%$ ) contain vector molecules. Vector replicons are packaged by the sPs, supplied in *trans* by the PCL, following vector RNA  
20 amplification, which proceeds according to the Sindbis virus replication strategy. In turn, the produced recombinant vector particles infect the remaining cells of the culture. Thus, a bloom of recombinant protein expression results over time as recombinant vector particles are produced and subsequently infect all cells in the PCL culture. Similarly, amplification of vector particles with PCL may be used to generate large,  
25 high titer particle stocks for other applications. In yet another aspect of this invention, recombinant protein expression from producer cell lines is described (*see* Example 7). Briefly, cell lines are derived which contain all of the genetic elements, including vector replicon and defective helper expression cassettes, from which the production of vector particles can be induced, via addition of an extracellular stimulus to the culture. Thus,  
30 expression of vector-encoded recombinant protein occurs as a result of induction of

alphavirus vector particle producer cell lines. In yet a still further aspect of this invention, recombinant protein expression from cell lines stably transformed with eukaryotic layered vector initiation systems are described (see Example 7). Briefly, cell lines are derived which are stably transformed with an inducible eukaryotic layered  
5 vector initiation system cassette that encodes a recombinant protein of interest. Thus, expression of vector-encoded recombinant protein occurs as a result of induction of the eukaryotic layered vector initiation system cassette.

As should be readily understood given the disclosure provided herein, protein production utilizing RNA vectors replicons, eukaryotic layered vector initiation  
10 systems, or recombinant vector particles may also be accomplished by methods other than introduction into packaging or producer cell lines. For example, such vectors may be introduced into a wide variety of other eukaryotic host cell lines (e.g., COS, BHK, CHO, 293, or HeLa cells), as well as direct administration *in vivo* or to *ex vivo* cells, in order to produce the desired protein.

15

#### J. Deposit Information

The following materials have been deposited with the American Type Culture Collection:

20

<u>Deposit</u>	<u>Designation</u>	<u>Deposit Date</u>	<u>Accession</u> <u>No.</u>
Wild type Sindbis virus	CMCC #4639	April 2, 1996	VR-2526
SIN-1 Sindbis virus	CMCC #4640	April 2, 1996	VR-2527
pBG-SIN1 ELVS1.5 SEAP	CMCC #4641	April 2, 1996	97502

The above materials were deposited by Chiron Corporation with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland under the terms of the Budapest Treaty on the International Recognition of  
25 the Deposit of Microorganisms for purposes of Patent Procedure. The accession number is available from the ATCC at telephone number (301) 881-2600.



These deposits are provided as convenience to those of skill in the art, and are not an admission that a deposit is required under 35 U.S.C. § 112. The nucleic acid sequence of these deposits, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and should be referred to in the event of an error in the sequence described therein. A license may be required to make, use, or sell the deposited materials, and no such license is granted hereby.

The following examples are included to more fully illustrate the present invention. Additionally, these examples provide preferred embodiments of the invention and are not meant to limit the scope thereof. Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely reorganized manuals of molecular biology, such as, for example, "Molecular Cloning," Second Edition (Sambrook et al., Cold Spring Harbor Laboratory Press, 1987) and "Current Protocols in Molecular Biology" (Ausubel et al., eds. Greene Associates/Wiley Interscience, NY, 1990).

## EXAMPLES

### EXAMPLE 1

#### ISOLATION AND CHARACTERIZATION OF SIN1

5

Below, the identification and molecular characterization of a positive strand RNA virus which exhibits reduced inhibition of host macromolecular synthesis and is capable of establishing persistent infection in vertebrate cells, as compared to lytic, cytopathogenic wild type strains of the same virus, is described. For example, 10 Sindbis virus is used as a prototype representative of the Alphavirus genus.

A. Isolation, plaque purification, and characterization of SIN-1 from a wild-type Sindbis virus stock

The isolation, molecular cloning, and characterization of a Sindbis virus 15 variant strain is described. This strain is able to establish productive persistent infection in the absence of cytopathicity, but produce levels of virus equivalent to that of wild-type virus.

A high-titered ( $>10^8$  PFU/ml) wild-type stock obtained by infection of BHK cells (ATCC No. CCL-10) with Sindbis virus (CMCC #4639) at low MOI ( $\leq 0.1$ ). 20 To facilitate infection, the virus inoculum was contained in a volume just sufficient to cover the monolayer when added to the cells. BHK cells were maintained, and all virus dilutions were performed, in Eagle minimal essential medium supplemented with 10% fetal calf serum. Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere. Extensive CPE, as demonstrated by "rounding up", loss of adhesion, and increased light refraction of 25 individual cells within the monolayer, and additionally, the decreased overall cell density of the monolayer, was observed within 48 hours post infection (hpi). The cell culture fluids were collected, cell debris was removed by low speed centrifugation (4,000 rpm for 10 min at room temperature), and the virus stock was aliquoted and stored at -70°C. The titer of the Sindbis virus stock was determined by plaque assay as 30 described previously (Strauss et al., *Virology* 74:154-168, 1976). Briefly, chicken embryo fibroblasts (CEF) monolayers were infected with various dilutions of the virus

stock and the monolayer overlaid with media supplemented with 0.75% agarose. At 24-48 hpi plaques due to cell lysis were visualized and quantitated either directly or, alternatively, by staining with crystal violet after removing the agarose overlay. The virus titer was determined from samples infected with virus dilutions in which the  
5 plaques were accurately quantitated.

A Sindbis virus stock enriched for DI particles was obtained by repeated high MOI passage ( $\geq 5$ ) on BHK cells. BHK monolayers were infected initially with the Sindbis virus seed stock at an MOI = 5. The culture medium was collected and clarified by low speed centrifugation after complete cell lysis of the infected culture was  
10 observed (usually within 24 hpi). The clarified medium collected from the infected culture was then used to infect a fresh BHK monolayer. For example, 2 ml of the virus inoculum was added to a fresh BHK monolayer in a 10 cm petri dish. At 1 hpi, 8 ml of fresh medium was added to the virus-infected culture. As described above, the culture medium was collected and clarified after observation of complete cell lysis of the  
15 culture. This process was repeated until the rate at which cytopathogenicity in the BHK monolayer developed after infection was delayed until at least 4 days. The delay in the onset of cytopathogenicity after infection signifies the presence of a high level of DI particles in the virus preparation.

The presence of a high level of DI particles in the virus preparation  
20 derived from multiple serial undiluted passages of infected cell medium was determined by an interference assay or by RNA analysis of BHK infected cells. In the first method, a homologous interference assay was performed as a measure of the presence of DI particles. Briefly, BHK cells were infected alone at an MOI = 10 with the high-titered ( $>10^8$  PFU/ml) wild-type stock prepared as described above. At 16 hpi, the virus yield  
25 was determined by plaque assay, as described above. The virus yield from this experiment was typically  $1 \times 10^9$  to  $1 \times 10^{10}$  PFU/ml. In another experimental group, BHK cells were simultaneously coinfectd with the wild-type stock (MOI = 10) and the virus stock prepared from multiple serial undiluted passages of infected cell medium (MOI = 5). As before, the virus yield was determined at 16 hpi by plaque assay. If the  
30 virus stock from the second experimental group contains a high level of DI particles, the

virus yield will be at least 2-3 orders of magnitude lower than the first experiment (e.g.,  $\leq 1 \times 10^7$  PFU/ml).

As a more definitive method for demonstrating the presence of DI particles in the virus preparation, virus-specific RNA in BHK infected cells at 16 hpi was analyzed. Briefly, BHK cells were infected (MOI = 10) with the high-titered ( $>10^8$  PFU/ml) wild-type stock or with the virus stock containing DI particles. Mock-infected controls and infected cells were treated with dactinomycin (1 mg/ml) and labeled with [ $^3$ H]uridine (20 mCi/ml) from 1 to 16 hpi. RNA was isolated from infected and control cells by using RNazol B, as described by the manufacturer (Tel-Test, Inc., Friendswood, Texas). Alternatively, RNA was isolated with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, Ohio), or by conventional methods using phenol extraction of cells lysed in a buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.5) containing 0.5% Triton and 0.5% recrystallized naphthalene disulfonate, as described by Weiss et al. (*J. Virol.* 14:1189-1198, 1974). The RNAs were denatured with glyoxal and electrophoresed through 1.1% horizontal agarose gels prepared in 0.01M sodium phosphate buffer (pH 7.0), at 5V/cm (McMaster and Carmichael, *Proc. Natl. Acad. Sci. USA* 74:4835-4838, 1977). Alternatively, RNA can be electrophoresed through formaldehyde gels. Following electrophoresis, all moisture was removed from the gels under vacuum with a gel dryer, and the dried gels were treated for fluorography and exposed to film. Two RNAs, corresponding to the genomic and subgenomic species (42S and 26S, respectively), were observed in samples from BHK cells infected with the wild-type virus stock. In contrast, a large number of RNA species that are distinct from the standard viral 42S and 26S RNAs were observed in samples from BHK cells infected with the virus stock containing DI particles. Multiple RNAs corresponding to DI RNAs migrated predominantly at molecular weights smaller than the 26S RNA species from wild-type virus. An example of multiple RNAs in addition to the 42S and 26S species observed in BHK cells infected with a virus stock containing DI particles may be seen in Figure 3, lane 3, of Weiss et al. (*J. Virol.* 33:463-474, 1980).

A Sindbis virus variant strain which is able to establish productive persistent infection with decreased cytopathogenicity was isolated and molecularly

cloned from a virus stock enriched for DI particles. BHK cells were infected at high multiplicity (MOI = 5) with the Sindbis virus stock enriched for DI particles. Cytopathogenicity developed slowly compared to infection of BHK cells with wild-type virus; however, most cells were eventually lysed and detached from the plate. Cell debris and non-adherent cells were removed every two days by medium changes. Within two weeks after initial infection, separate and distinct colonies were observed. These colonies were thriving and demonstrated no morphological evidence of CPE, compared to uninfected BHK cell controls. Within 3-4 weeks, the cell colonies were large and discernible to the naked eye. The colonies were isolated with cloning rings, and the cells were dispersed with either 3 mM EDTA or trypsin. Dispersed cells from each colony were replated without dilution. Thereafter, cells were subcultured at a 1:10 dilution upon reaching confluency, generally within four days. Aliquots of cells, designated BHK(SIN-1), were prepared in cryotubes after the fifth passage for long term storage in liquid nitrogen. BHK(SIN-1) cells were indistinguishable from the original, uninfected BHK cells in terms of growth rate or morphology.

#### B. Molecular Cloning of SIN-1

To characterize the mutation(s) in the Sindbis genome which correlate with the development of the substantially reduced cytopathogenicity of SIN-1, genomic RNA from SIN-1 virions was isolated, reverse transcribed, and the resultant cDNA encompassing the nonstructural protein genes sequenced, as described more fully below.

Briefly, the SIN-1 virus was plaque purified three times before preparation of a stock that was used for the isolation of RNA. The BHK(SIN-1) cells were grown as described above, the culture fluid was collected, and various dilutions were used to infect primary chicken embryo fibroblast monolayers (CEF, grown in minimal essential medium supplemented with 3% fetal calf serum). Following infection, medium containing 0.5% noble agar was added to the monolayers. Additionally, DEAE-dextran (100 µg/mL) can be included in the agar-overlay medium to increase the size of the SIN-1 plaques. Individual discreet plaques were observed after 3 days of incubation at 30°C in plates infected with suitably dilute inoculums of

the BHK(SIN-1) culture fluid. After the third round of purification, the cloned SIN-1 virus was passaged once at 30°C in CEF cells infected at a MOI = 0.1. The plaque-purified SIN-1 virus preparations were determined to be free of DI particles by the interference assay and RNA analysis in BHK infected cells, as described above.

- 5 BHK cells were infected (MOI = 10) with the plaque purified SIN-1 stock to determine the ability of this wild-type Sindbis virus variant strain to establish persistence. As described above, establishment of persistent infection in BHK cells with wild-type Sindbis virus requires the presence of DI particles in the virus preparation and considerable time to allow those few surviving cells to grow out. In  
10 contrast, persistent infection was readily established in BHK cells infected at 37°C with the SIN-1 variant whether or not DI particles are present in the virus inoculum. At six days post infection with SIN-1, the BHK cells were completely resistant to superinfection with wild-type Sindbis virus, demonstrating establishment of a persistent infection. However, these cells were susceptible to infection by the heterologous virus,  
15 VSV, demonstrating that interferon is not involved in the establishment of SIN-1 persistent infection.

- CEF cells were infected (MOI = 10) with the plaque purified SIN-1 stock to generate a high titered stock of virus for the isolation of RNA. Ninety ml of culture fluid ( $2 \times 10^{10}$  PFU/ml) was clarified by centrifugation for 5 min at 2,500 rpm in a Sorvall  
20 GSA rotor. The SIN-1 virus was pelleted from the clarified culture fluid by centrifugation in an SW27.1 rotor for 2h at 24,000 rpm, at 4°C. The virus pellet was then resuspended in 3 ml of culture media, homogenized by repeated pipetting, and layered on top of a 25-40% (w/w) sucrose gradient. This was followed by centrifugation in an SW27.1 rotor for 4 h at 24,000 rpm, at 4°C. The virus band was  
25 visualized with incandescent light illumination, and collected with a 22 gauge needle/syringe. The RNA was purified further by Proteinase K (Boehringer Mannheim, Indianapolis, Indiana) digestion (56°C, 1 hr), followed by extraction with an equal volume of H<sub>2</sub>O-saturated phenol:chloroform (1:1 v/v, pH 7.0), followed by precipitation with 2 volumes of ethanol and 0.3 M sodium acetate, pH 5.2.

Alternatively, the SIN-1 virus can be further purified by polyethylene glycol precipitation of infected BHK cell culture media (Strauss et al. *Virology* 133:154-168, 1976), or alternatively by pelleting through a sucrose cushion (Polo et al. *J. Virol.* 62:2124-2133, 1988).

5 Two rounds of first strand cDNA synthesis were performed with the purified viral RNA, using the Moloney murine leukemia virus or SuperScript II reverse transcriptases (Gibco-BRL, Gaithersburg, Maryland) according to the manufacturer's recommended conditions. Six separate reactions were performed, using the Sindbis virus specific primers complementary to the positive strand (primers denoted by R)  
10 shown below. All primers denoted by R were phosphorylated at their 5' end with polynucleotide kinase (New England Biolabs) prior to the first strand synthesis reaction to facilitate cloning.

Primer	Location	Seq. ID No.	Sequence (5' -> 3')	Enzyme Site
T7/1F	<i>Sac</i> I/T7/1-20	2	GGTGGAGCTCTAATACGACT CACTATAGATTGACGGCGTA GTACACAC	<i>Sac</i> I
1465R	1465-1451	3	AATTTCTGCCTCAGC	<i>Eco</i> 47 III
1003F	1003-1019	4	TATGCAAAGTTACTGAC	<i>Eco</i> 47 III
2823R	2823-2806	5	CTGTCATTACTTCATGTC	<i>Bsp</i> HI
1003F	1003-1019	4	TATGCAAAGTTACTGAC	<i>Eco</i> 47 III
4303R	4303-4289	6	GCGTGGATCACTTTC	<i>Avr</i> II
4051F	4051-4069	7	ATTGCGTGATTTCGTCCGT	<i>Avr</i> II
8115R	8115-8101	8	TAAATTTGAGCTTTG	<i>Pml</i> I
1680F	1680-1689	9	GGCATATGGCATTAGTTG	<i>Bsp</i> HI
8115R	8115-8101	8	TAAATTTGAGCTTTG	<i>Pml</i> I
8034F	8034-8052	10	CTGGCCATGGAAGGAAAGG	<i>Pml</i> I
11,703R	<i>Xho</i> I/dT <sub>21</sub> / 11703-11677	11	CCCCTCGAGGGT(21)GAAATG TTAAAAACAAAATTTTGTTG	<i>Xho</i> I

Synthesis of the second strand from the cDNA template above was accomplished in six separate reactions with the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA) according to the manufacturer's recommended conditions. Sindbis virus specific primers complementary to the negative strand (primers denoted by F shown above) were used. The double-stranded DNA products were substituted, stepwise, for the corresponding regions in the plasmid Totol101, which contains the full-length Sindbis virus genome (Rice et al., *J. Virol*, 61:3809-3819, 1987). For example, the T7/1F-1465R product was digested with *Sac* I and *Eco* 47III, and inserted into *Sac* I/*Eco* 47III digested and CIAP treated Totol101 plasmid, which was purified away from the *Sac* I/*Eco* 47III small fragment (SP6 promoter, Sindbis virus nts. 1-1407) by 1% agarose/TAE (50X/liter: 242 g Tris base/57.1 ml glacial acetic acid/100 ml 0.5 M EDTA pH 8.0) electrophoresis, and GENECLAN II. This



construct was then digested with *Eco* 47III and *Avr* II (Sindbis virus nt nos. 1407 and 4281, respectively), treated with CIAP, and followed by insertion of the 3300 bp fragment isolated from the 1003F-4303R product, digested with *Eco* 47 III and *Avr* II. The fully assembled clone is designated as pRSIN-1g (g, as a reference to full-length genomic clone), and contained all 11,703 bp of viral genome. A subset of the primers listed in the table above generate redundant double-stranded DNA reaction products within the SIN-1 genome. For example, the sequences in the 4051F/8115R product are within the 1680F/8115R product. These redundant products are provided as construction alternatives for the SIN-1 genomic clone; *i.e.*, in general, the efficiency of cDNA cloning is inversely proportional to the length of the desired fragment.

To clone portions of the viral genome not obtained by the above method, the SIN-1 RNA viral genome was cloned by reverse transcription polymerase chain reaction (RT-PCR). First strand synthesis was accomplished as described above. PCR amplifications of Sindbis cDNA with the primer pairs shown above were performed as separate reactions, using the KlenTaq1 enzyme, and the reaction conditions, as described in Barnes (*Proc. Natl. Acad. Sci. USA* 91:2216-2220, 1994). Alternatively, the Thermalase thermostable DNA polymerase (Amresco Inc., Solon, OH) was substituted for the KlenTaq 1 enzyme, using a buffer containing 1.5 mM MgCl<sub>2</sub> that was provided by the supplier. Alternatively, the VentR thermostable DNA polymerase (New England Biolabs, Beverly, MA) was used in the amplification reactions. Additionally, the reactions contained 5% DMSO and "HOT START WAX" beads (Perkin-Elmer). The PCR amplification protocol used is shown below (The 72°C extension incubation period was adjusted to 1 min per 1 kb of template DNA):

	Temperature (°C)	Time (Min.)	No. Cycles
(a)	95	2	1
(b)	95	0.5	35
	55	0.5	
	72	3.5	
(c)	72	10	1

Alternatively, cloning of the full-length SIN-1 RNA genome can be performed similar to methods which have previously been described (Dubensky et al., *J. Virol.* 70:508-519, 1996). Briefly, first strand cDNA synthesis is accomplished with a mixture of random hexamer primers (50 ng/ml reaction concentration) (Invitrogen, San Diego, California), and primer 4B, whose sequence is shown in the table below. Genomic length Sindbis virus SIN-1 variant cDNA is then amplified by PCR. Six distinct segments using six pairs of overlapping primers is sufficient to clone the entire genome. In addition to viral complementary sequences, the SIN-1 5' end forward primer contains a 19 nucleotide sequence corresponding to the bacterial SP6 RNA polymerase promoter and the *Apa* I restriction endonuclease recognition sequence linked to its 5' end. The bacterial SP6 RNA polymerase is poised such that transcription *in vitro* results in the inclusion of only a single non-viral G ribonucleotide linked to the A ribonucleotide, which corresponds to the authentic Sindbis virus 5' end. Inclusion of the *Apa* I recognition sequence facilitates insertion of the PCR amplicon into the plasmid vector pKS II<sup>+</sup> (Stratagene, La Jolla, California) polylinker sequence. A five nucleotide 'buffer sequence' extension is also linked upstream from the *Apa* I recognition sequence in order to facilitate efficient enzyme digestion. The sequence of the SP6-5' SIN-1 forward primer and all of the primer pairs necessary to amplify the entire SIN-1 genome are shown in the table below. (Note that "nt" and "nts" as utilized hereinafter refer to "nucleotide" and "nucleotides," respectively). The reference sequence (GenBank accession no. J02363, locus: SINCG) is from Strauss et al., *Virology* 133:92-110, 1984.

Primer	Location	Seq. ID No.	Sequence (5'→3')	Recognition Sequence
SP6-1A	<i>Apa</i> I/SP6/ SIN nts.1-18	12	TATATGGGCCCCGATTTAGGTGAC ACTATAGATTGACGGCGTAGTAC AC	<i>Apa</i> I
1B	3182-3160	13	CTGGCAACCGGTAAGTACGATAC	<i>Age</i> I
2A	3144-3164	14	ATACTAGCCACGGCCGGTATC	<i>Age</i> I
2B	5905-5885	15	TCCTCTTTCGACGTGTCGAGC	<i>Eco</i> RI
3A	5844-5864	16	ACCTTGGAGCGCAATGTCCTG	<i>Eco</i> RI
7349R	7349-7328	17	CCTTTTCAGGGGATCCGCCAC	<i>Bam</i> HI
7328F	7328-7349	18	GTGGCGGATCCCCTGAAAAGG	<i>Bam</i> HI
3B	9385-9366	19	TGGGCCGTGTGGTTCGTCATG	<i>Bcl</i> I
4A	9336-9356	20	TGGGTCTTCAACTCACCGGAC	<i>Bcl</i> I
10394R	10394-10372	21	CAATTCGACGTACGCCTCACTC	<i>Bsi</i> WI
10373F	10373-10394	22	GAGTGAGGCGTACGTCGAATTG	<i>Bsi</i> WI
4B	<i>Xba</i> I/T <sub>35</sub> / 11703-11698	23	TATATTCTAGA(T <sub>35</sub> )GAAATG	<i>Xba</i> I

PCR amplifications of Sindbis cDNA with the primer pairs shown above are performed as separate reactions, using the Thermalase or Vent<sub>R</sub> DNA polymerases (cited above), reaction conditions, and the PCR amplification conditions, as described above.

The regions of sequence overlap between the amplification products correspond to unique enzyme recognition sites within the PCR amplicon. The PCR products are purified (QIAquick PCR purification kit, Qiagen, Chatsworth, California) and inserted stepwise into the pKS II<sup>+</sup> vector, between the *Apa* I and *Xba* I sites. The fully assembled clone is designated as pKSRSIN-1g (g, as a reference to full-length genomic clone), and contains all 11,703 bp of viral genome.

### C. Sequence of the SIN-1 phenotype

The SIN-1 specific nucleotide sequences of the pRSIN-1g clone was determined by the dideoxy-chain termination method. Sequence comparison of 8,000 bp of viral sequence revealed multiple differences between the SIN-1 clone described herein and the Sindbis virus (strain HRsp) sequence provided in GenBank (GenBank

Accession no. J02363, locus: SINCG). Differences in the sequence among SIN-1 (Figure 6), SINCG (Figure 7), and Toto1101 (Figure 8) are presented below.

nt. Position	Gene	SINCG		Toto 1101		SIN-1	
		nt	aa	nt	aa	nt	aa
45	5' NTR	T	--	T	--	C	--
120	nsP1	C	Gln	C	Gln	A	Lys
1775	nsP2	G	null	G	null	A	null
1971	nsP2	T	Phe	T	Phe	C	Leu
2992	nsP2	C	Pro	T	Leu	T	Leu
3579	nsP2	A	Lys	G	Glu	G	Glu
3855	nsP2	C	Pro	C	Pro	T	Ser
3866	nsP2	C	null	C	null	T	null
4339	nsP3	A	Glu	A	Glu	T	Val
4864	nsP3	C	Ser	C	Ser	T	Phe
5702	nsP3	A	null	T	null	T	null
5854*	nsP4	G	Arg	G	Arg	A	His
7612	junction	A	--	A	--	T	--
7837	Capsid	C	Arg	C	Arg	T	Cys

- 5 \* This mutation was found in one cDNA clone. It was not detected when the Sin-1 virus RNA was sequenced. It likely represents a minor species in the RNA population.

Verification that the sequence changes were unique to the clone (and not the result of cloning artifact) described herein, was determined by amplifying SIN-1 virion RNA by RT-PCR as described above, establishing the sequence containing the nucleotides in question by direct sequencing of the RT-PCR amplicon product, and comparing the sequence to the corresponding SIN-1 sequence.

15 D. Characterization and genetic mapping of the SIN-1 phenotype with molecular clones:

Various regions of the SIN-1 genome were substituted for the corresponding wild-type Sindbis virus region in the Toto1101 plasmid (Rice et al., J.

*Viol.* 61:3809-3819, 1987) in order to map the location of the phenotype for establishment of persistence. The various SIN-1 nsP genes were substituted into the Toto 1101 wild-type Sindbis virus background using restriction enzyme fragments purified from pRSIN-1g, as illustrated in the table below.

5

nsP Gene	Restriction Fragment	Nucleotide Coordinates	Clone Designation
nsP1	<i>Ple</i> I/ <i>Eco</i> 47III	98-1407	pRSIN-1nsP1
nsP2	<i>Eco</i> 47III/ <i>Avr</i> II	1407-4281	pRSIN-1nsP2
nsP2-N terminus	<i>Eco</i> 47III/ <i>Bgl</i> II	1407-2289	pRSIN-1nsP2-N
nsP2-C terminus	<i>Bgl</i> II/ <i>Avr</i> II	2289-4281	pRSIN-1nsP2-C
nsP3-4	<i>Avr</i> II/ <i>Eco</i> RI	4281-5870	pRSIN-1nsP3
nsP3	<i>Avr</i> II/ <i>Spe</i> I	4281-5262	pRSIN-1nsP3
nsP4	<i>Spe</i> I/ <i>Aat</i> II	5263-5870	pRSIN-1nsP4
nsP1-4	<i>Ple</i> I/ <i>Aat</i> II	98-8000	pRSIN-1nsP1-4

The coordinates of the nonstructural gene coding regions are provided in the following table:

nsP Gene	Coordinates of Sindbis virus genome (nt. no.)
nsP1	60-1680
nsP2	1680-4101
nsP3	4101-5769
nsP4	5769-7597
nsP1-4	60-7597

10

The various SIN-1, Toto, and chimeric SIN-1/Toto clones, pRSIN-1g, Toto, pRSIN-1nsP1, pRSIN-1nsP2, pRSIN-1nsP2-N, pRSIN-1nsP2-C, pRSIN-1nsP3, pRSIN-1nsP4, and pRSIN-1nsP1-4 were linearized by digestion with *Xho* I, which

makes a single cut in the cDNA clones immediately adjacent and downstream of a 21 nucleotide poly dA:dT tract following the Sindbis virus 3' end (viral nt. 11703). The linearized clones were purified with GENECLAN II (BIO 101, La Jolla, California), and adjusted to a concentration of 0.5 µg/µl. Transcription of the linearized clones was

5 performed *in vitro* at 40°C for 90 minutes according to the following reaction conditions: 2 µl DNA/4.25 µl H<sub>2</sub>O); 10 µl 2.5 mM NTPs (UTP, ATP, GTP, CTP); 1.25 µl 20 mM Me7G(5')ppp(5')G cap analogue; 1.25 µl 100 mM DTT; 5 µl 5X transcription buffer (Promega, Madison Wisconsin); 0.5 µl RNasin (Promega); 0.25 µl 10 µg/µl bovine serum albumin; and 0.5 µl T7 RNA polymerase (Promega). The

10 *in vitro* transcription reaction products were digested with DNase I (Promega), purified by sequential phenol:CHCl<sub>3</sub> and ether extraction, and followed by ethanol precipitation. Alternatively, the *in vitro* transcription reaction products can be used directly for transfection. The *in vitro* transcription reaction products or purified RNA were complexed with a commercial cationic lipid compound (LIPOFECTIN, GIBCO-BRL,

15 Gaithersburg, MD) and applied to Baby Hamster Kidney-21 (BHK-21) cells maintained in a 60 mm petri dish at 75% confluency. Alternatively, BHK cells were electroporated with the *in vitro* transcription reaction products or purified RNA, exactly as described previously (Liljestrom, *Bio/Technology* 9:1356-1361, 1991). The transfected cells were incubated at 37°C. At 48 hours post-transfection, culture media were collected and the

20 titer of each virus was determined by plaque assay, as described above. The titered virus stocks derived from these *in vitro* transcription reactions were designated as shown in the table below.

Clone Designation	Virus Designation
pRSIN-1nsP1	SIN-1nsP1
pRSIN-1nsP2	SIN-1nsP2
pRSIN-1nsP2-N	SIN-1nsP2-N
pRSIN-1nsP2-C	SIN-1nsP2-C
pRSIN-1nsP3-4	SIN-1nsP3-4
pRSIN-1nsP3	SIN-1nsP3
pRSIN-1nsP4	SIN-1nsP4
pRSIN-1nsP1-4	SIN-1nsP1-4
Toto 1101	Toto

To map the SIN-1 persistent phenotype,  $8 \times 10^5$  BHK cells were infected (MOI=5) with each of the virus stocks prepared above. At 3 days post infection, the culture viability was determined by trypan blue dye exclusion. The results of this experiment (shown below), demonstrate that the SIN-1 phenotype of establishing persistent non-cytocidal infections maps to the nonstructural genes, and to nsP2 gene in particular. The number of cells in the mock-infected culture represents continued growth of these cells until they reached the stationary phase. At 3 dpi, cells infected with SIN-1nsP1, SIN-1nsP3, SIN-1nsP3-4 and SIN-1nsP4 had all died. The cells that survived infection with SIN-1nsP2 and SIN-1nsP1-4 continued to grow and were persistently infected based on staining with antibodies specific for Sindbis virus.

Virus	Number of Cells at 3 dpi
SIN-1nsP1	0
SIN-1nsP2	$3 \times 10^5$
SIN-1nsP3-4	0
SIN-1nsP3	0
SIN-1nsP4	0
SIN-1nsP1-4	$5 \times 10^5$
Toto	0
Mock	$1 \times 10^7$

As shown above, the observed SIN-1 phenotype of establishing non-cytocidal persistent infections maps to the viral nsPs, as opposed to the sPs. This conclusion was demonstrated clearly by comparison of cell survival levels between

5 cultures infected with the Toto or SIN-1nsP1-4 virus stocks. Both the Toto and SIN-1 nsP1-4 viruses contain the wild-type sPs; cell survival was observed, however, only in those cultures infected with the virus (SIN-1nsP1-4) containing nsPs derived from the SIN-1 clone. In these experiments, cell survival was not dependent upon the source of the Sindbis virus sPs. Importantly, the SIN-1 phenotype was mapped further to nsP2.

10 The level of cell survival was comparable between cultures infected with the SIN-1nsP1-4 or SIN-1nsP2 viruses. Further, a C  $\rightarrow$  T transition at nucleotide 3855, in the SIN-1 nsP2 gene is responsible for the characteristic phenotype of establishment of persistent infection in cells infected with the SIN-1 virus. The single proline to serine change in the nsP2 protein produced in cells infected with the chimeric virus SIN-

15 1nsP2-C, was all that was required to convert wild-type Sindbis virus (Toto 1101) from a virus that killed all of the infected cells into a virus which permitted many of the infected cells to survive and continue to produce virus. The phenotypes of chimeric viruses derived from insertion of the SIN-1 nsP1, nsP3, or nsP4 genes into the Toto background were indistinguishable from wild-type and complete lysis was observed in

20 cultures infected with these viruses.



The possible effect of amino acid changes in the SIN-1 nsPs on the level of productive infection in BHK cells was determined by comparing the virus yield over a time course in BHK cells inoculated with the various SIN-1, wild-type (Toto), or SIN-1/Toto chimeric strains. Briefly, BHK cells were infected (MOI=20) with SIN-1 (plaque purified stock described above), Toto, SIN-1nsP1-4, or SIN-1nsP2 viruses, and the culture fluids were collected at 3, 6, 9, and 12 hours post infection. The titers of virus in the culture fluids were then determined by plaque assay, as described above. The results of this study, shown in Figure 2, demonstrate that equivalent levels of virus were produced in BHK cells infected with wild-type or SIN-1 strains. The actual virus titers at the 12 hpi time point are set forth in the table below. More than half of the BHK cells survived infection with SIN-1 virus (and chimeric viruses containing SIN-1 nsP1-4, or SIN-1 nsP2) in combination with levels of virus production equivalent to wild-type strains.

Virus	Titer (PFU/ml) at 12 hpi ( $\times 10^9$ )
SIN-1	3.6
Toto	2.7
SIN-1nsP1-4	1.8
SIN-1nsP2	2.6

15

The possible effect of amino acid changes in the SIN-1 nsPs on the level of viral-specific RNA synthesis was determined by comparing the level of [ $^3$ H]-uridine incorporation over a time course in BHK cells inoculated with the various SIN-1, wild-type (Toto), or SIN-1/Toto chimeric strains. BHK cells ( $3 \times 10^5$  cells/35 mm dish) were grown at 37°C, according to the conditions described herein. The cells were infected (MOI = 20) with SIN-1, Toto1101, SIN-1nsP1-4, or SIN-1nsP2 viruses. At 30 min. post infection, the culture medium was adjusted to 1  $\mu$ g/ml actinomycin D. After incubation for an additional 30 min, the culture medium was adjusted to 10  $\mu$ Ci/ml [ $^3$ H]-uridine. At 3, 6, 9, and 12 hpi, the treated cells were washed with PBS, and lysed by addition of 200  $\mu$ l of TTE buffer (0.2% Triton X-100, 10 mM Tris-HCl, pH 8.0, 1

25

mM EDTA). The RNA was precipitated at 4°C by addition of 200 µl of a 25% trichloroacetic acid (TCA) solution. The RNA was pelleted by microcentrifugation for 5 min at 14,000 rpm, rinsed once with 5% TCA, and dissolved in a solution consisting of 50 mM NaOH/0.1 % SDS, at 55°C. The solution containing the dissolved RNA was transferred into scintillation vials and the level of incorporated [<sup>3</sup>H]-uridine was determined using EcoLume (ICN, Irvine, CA) scintillation fluid. The results of this study, shown in Figure 3, demonstrate that levels of virus-specific RNA were dramatically lower in BHK cells infected with SIN-1 compared to wild-type virus. This phenotype of low level of virus-specific RNA synthesis maps to the nsPs, as shown by the equivalently low levels of RNA produced in BHK cells infected with the SIN-1 or SIN-1nsP1-4 strains, compared to wild-type.

Virus	[ <sup>3</sup> H]uridine incorporation (x10 <sup>3</sup> cpm)
SIN-1	22
Toto1101	86
SIN-1nsP1-4	28
SIN-1nsP2	62
Mock	8

Virus-specific RNA synthesis in infected BHK cells was also determined using all of the SIN-1, Toto, and SIN-1/Toto chimeric, strains. The levels of [<sup>3</sup>H]uridine incorporation, relative to wild-type infection (Toto) at 9 hpi, are shown in Figure 4, and given in the table below.

Virus	No. of Experiments	Relative RNA Synthesis Level	Standard Deviation
Toto1101	9	1.0	
SIN-1	9	0.1	$\pm 0.1$
SIN-1nsP1-4	8	0.2	$\pm 0.1$
SIN-1nsP1	7	1.0	$\pm 0.2$
SIN-1nsP2	8	0.6	$\pm 0.1$
SIN-1nsP3	4	1.0	$\pm 0.0$
SIN-1nsP3-4	7	0.4	$\pm 0.1$
SIN-1nsP4	6	0.8	$\pm 0.1$
SIN-1nsP2-N	1	0.9	
SIN-1nsP2-C	3	0.6	$\pm 0.2$
Mock	9	0.0	

Thus, BHK cells survive infection with SIN-1 virus (and chimeric viruses containing SIN-1 nsPs1-4, or SIN-1 nsP2), SIN-1 virus levels equivalent to wild-type strains are produced in BHK cells, and, the level of viral-specific RNA synthesized is 10-fold lower compared to wild-type virus.

- The possible effect of amino acid changes in the SIN-1 nsPs on the level of inhibition of host cell protein synthesis was determined by comparing the level of protein synthesis, relative to uninfected cells, over a time course in BHK cells inoculated with the various SIN-1, wild-type (Toto), or SIN-1/Toto chimeric strains.
- 10 Briefly, 35 mm dishes seeded with  $2 \times 10^5$  BHK cells were infected (MOI = 20) with the various Sindbis virus strains in a 0.5 ml virus inoculum in a buffer consisting of PBS/1% fetal calf serum. The plates were incubated at 4°C for 1 hr with continuous gentle shaking. The inoculum was replaced with 2 ml of medium, described previously, containing 10% fetal calf serum, and the dishes were placed in a CO<sub>2</sub> incubator at 37°C.
- 15 At 5, 8, and 11 hr later, the media was replaced with 2 ml of MEM lacking methionine (Met-), with 2% fetal calf serum, and incubated 30 min. The medium was then replaced with 1 ml of MEM (Met-) containing 2% fetal calf serum and 10  $\mu$ Ci/ml of [<sup>35</sup>S]methionine. Following a 30 min incubation period at 37°C, 1 ml of medium

containing 10% fetal calf serum was added to each well, and the dishes were incubated for another 30 min at 37°C. This dilution is sufficient to inhibit further incorporation of radioactive label into protein and significantly decreases the background of free [<sup>35</sup>S]-methionine detected in polyacrylamide gels. The medium was then removed from the well. Cells were washed three times with PBS, scraped from the dish into PBS, pelleted by centrifugation, and dissolved in 25 µl of loading buffer (0.06 M Tris-HCl, pH 6.7, 2% SDS, 5% β-mercaptoethanol, 5% glycerol, 0.05% bromophenol blue). One-fifth of the sample was analyzed on the gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R, dried, and autoradiographed. The rates of inhibition of host cell protein synthesis were compared by quantitating the amount of radioactivity in the section of the gel containing only host proteins. The results of this study are shown in Figure 5, and demonstrate that the level of inhibition of host cell protein synthesis is significantly lower in SIN-1 virus infected cells, compared to wild-type virus infected cells, particularly at the earlier 6 and 9 hour time points post infection.

In summary, BHK cells survive SIN-1 infection, virus levels equivalent to wild-type strains are produced in BHK cells, the level of viral-specific RNA synthesized is 10-fold lower than wild-type virus, and the level of inhibition of host cell protein synthesis in SIN-1 virus infected cells is significantly lower compared to wild-type virus infected cells. The phenotypes of the SIN-1 virus described herein map to the viral nsP genes.

## EXAMPLE 2

### ISOLATION AND CHARACTERIZATION OF POSITIVE STRAND RNA VIRUSES WHICH EXHIBIT REDUCED INHIBITION OF HOST MACROMOLECULAR SYNTHESIS

The derivation of virus variants exhibiting the desired phenotypes of reduced, delayed or no inhibition of host cell macromolecular synthesis is dependent on the generation, characterization, and isolation of sequences which differ from that of wild-type virus. However, with the exception of example 1, there are no obvious or

previously disclosed methods to select for or identify coding or non-coding viral sequence changes that result in alteration of this virus-based inhibition of macromolecular synthesis, or the generation of viruses that lead to persistent, rather than lytic, infection. This example provides specific methods and that enable one to  
5 overcome these obstacles.

A. Biological Selection of Virus Variants

The biological derivation of virus variants which result in reduced, delayed, or no inhibition of host macromolecular synthesis, or which establish persistent  
10 infections, can be performed by allowing for natural, spontaneous mutation within a cell, or first subjecting the desired virus stock to physical, chemical or other artificial mutagenesis, followed by infection of susceptible cells, and successive enrichments for those cell populations which harbor mutated virus. It is possible that prior mutagenesis, although not required, will facilitate the generation of appropriate mutations. The  
15 selection is based on the ability of cells infected with the desired variant to survive for significantly longer periods than wild-type virus infected cells. The following example provides methods in detail, using Sindbis virus as an example; however, other viruses are readily substituted, as noted in the detailed description.

Specifically, in the case of chemical mutagenesis, a Sindbis virus stock  
20 suspension with a titer of greater than or equal to  $10^9$  pfu/ml is treated with nitrosoguanidine at a final concentration of 100 ug/ml. After 15 minutes at room temperature, the nitrosoguanidine is removed by dialysis at 4°C and the mutagenized stock is subsequently used for infection. Approximately  $5 \times 10^6$  cells of the desired type (for example BHK-21), grown in flat stock culture, are infected with the mutagenized  
25 virus at a multiplicity of infection (M.O.I.) of approximately 5, to ensure that every cell is infected. At 12, 24, 36, and 48 hours post-infection, the cell monolayer is washed twice with fresh media to remove dead cells, and replaced with media consisting of a mixture of 50% fresh media and 50% conditioned media. After a desired time post-infection (for example 72 hours), the remaining cells are gently trypsinized to detach  
30 them from the culture dish and strip away any cell-associated extracellular virus, which is then separated from the cells by differential centrifugation. The remaining cells are

then re-seeded directly into a tissue culture dish containing a semi-confluent uninfected cell monolayer, at a ratio of 1 infected cell for every  $10^3$  uninfected cells. Additional rounds of selection are performed by seeding onto uninfected cells for amplification, followed by the above washing and harvesting steps. Alternatively, the initial  
5 infections may be done using a wild-type virus stock at low M.O.I., allowing for spontaneous mutation during replication within the cell. Using this approach, a heterogenous population of mutant virus is produced by the infected cells. In those instances where the population of infected cells recovered after trypsinization includes a significant number of non-viable or severely damaged cells, a brief treatment (5  
10 minutes at room temperature) with 0.75%  $\text{NH}_4\text{Cl}$  in 17 mM Tris (pH 7.65 with HCl), or centrifugation through Percoll™, is included to remove remaining dead or damaged cells, prior to re-seeding onto uninfected cell monolayers. After a minimum of two successive rounds of selection, virus variants displaying the desired phenotype are isolated by limiting dilution or plaque purification, and subjected to cDNA cloning as  
15 described in Example 1. Isolation and characterization of specific sequences responsible for the variant phenotype are accomplished by substitution of defined regions of cDNA into a genomic clone or expression vector and testing for the accompanying phenotypic change, as outlined in the Examples.

20 B. Genetic Selection of Virus Variants

In a related approach, natural mutation or random mutagenesis is performed, not on a virus stock, but rather, using cloned genomic cDNA of the virus that can be transcribed into infectious viral RNA *in vitro* or *in vivo*. For example, in the case of prior mutagenesis, plasmid pRSING, which contains a full-length genomic  
25 Sindbis cDNA functionally linked to a bacteriophage SP6 promoter (Dubensky, et al., *J. Virol.* 70:508-519, 1996), is transformed into competent *E. coli* XL1-Red mutator cells and plated on ampicillin plates to obtain colonies. At least 200 colonies are chosen at random, pooled, and inoculated for overnight growth in a 10 ml broth culture containing ampicillin. Plasmid DNA is prepared from the culture to obtain a  
30 heterogeneous population of pRSING harboring various mutations. The DNA is linearized with *Xba* I and transcribed *in vitro* using SP6 polymerase, as described

previously (Rice et al., *J. Virol.* 61:3809-3819, 1987). RNA transcripts are subsequently transfected into the desired cell type (for example, BHK cells) by electroporation (Liljestrom and Garoff, *Bio/Technology* 9:1356-1361, 1991), for initiation of the Sindbis virus infection cycle. Alternatively, *in vitro* transcribed RNA  
5 from an unmutagenized template may also be transfected. Selection of virus mutants which establish a persistent infection or exhibit reduced, delayed, or no inhibition of host macromolecular synthesis is performed as above. The selected virus variants with the desired phenotype are isolated by limiting dilution or plaque purification, subjected to cDNA cloning, and the sequences responsible for the variant phenotype are isolated  
10 and characterized, as described previously.

### C. Genetic Selection of Variants Using Virus-Derived Vectors

#### 1. Vectors Expressing an Immunogenic Protein

In another approach, spontaneous intracellular mutation, or random  
15 mutagenesis is performed on virus-derived sequences of a viral-based expression vector. These sequences include non-coding and regulatory regions, as well as nonstructural protein encoding regions. In certain instances, structural protein-encoding sequences also may be included. Such random mutagenesis or spontaneous intracellular mutation may be performed using any of the techniques described in this invention, along with  
20 the cloned cDNA of a virus-derived vector which can be transcribed into RNA *in vitro* or *in vivo*. For example, a replication-competent Sindbis virus expression vector can be used to express an immunogenic cell surface protein or other peptide which may be bound by specific antibodies added to the infected cells. Cells which contain functional vector are identified by their expression of the vector-encoded heterologous antigen and  
25 ability to be bound by antibody specific for the encoded antigen. By limiting the selection process to cells surviving for extended periods (*see above*), only those harboring vector variants exhibiting the desired phenotype are enriched.

Specifically, plasmid pTE3'2J (Hahn et al., *J. Virol.* 89:2679-2683, 1992), comprising an SP6 promoter operably linked to a full-length genomic Sindbis  
30 cDNA with a duplicated subgenomic promoter for expression of heterologous genes, is subjected to random mutagenesis, as described above. This process results in isolation

of a population of heterogeneous plasmid containing the random mutations. In parallel, the desired heterologous cell surface protein or marker peptide gene is cloned into a shuttle vector for insertion into the mutagenized pTE3'2J vector. Preferred cell surface proteins for use as markers include human B7.1 (Freeman et al., *J. Immunol.* 143:2714-2722, 1989) and the murine H-2K<sup>b</sup> class I molecule (Song et al., *J. Biol. Chem.* 269:7024-7029, 1994). The human B7.1 gene is amplified by standard three-cycle PCR, with 1.5 minute extension, from a pCDM8 vector containing the full-length cDNA sequence (Freeman et al., *ibid*), using the following oligonucleotide primers that are designed to contain flanking *Xba* I and *Bam* HI sites.

10

Forward primer: hB7.1 FX (5'-rest. site/B7.1 sequence) (SEQ. ID. NO. 24)

5'-ATATATCTAGA/GCCATGGGCCACACACGGAGGCAG-3'

Reverse primer: hB7.1 RB (5'-rest. site/B7.1 sequence) (SEQ. ID. NO. 25)

15 5'-ATATAGGATCC/CTGTTATACAGGGCGTACACTTTC-3'

Following amplification, the approximately 875 bp DNA fragment is purified using a QIAquick-spin PCR purification kit (Qiagen, Chatsworth, CA), digested with *Xba* I and *Bam* HI and ligated into Sindbis shuttle vector pH3'2J (Hahn et al., *ibid*) that also has been digested with *Xba* I and *Bam* HI and treated with calf intestinal alkaline phosphatase, to create the construct pH3'B7.1.

Following random mutagenesis of the pTE3'2J double subgenomic vector, as described above, plasmid pH3'B7.1 and the mutated population of plasmid pTE3'2J are digested with *Apa* I and *Xho* I, purified from 0.7% agarose gels using GENECLAN II II™ (Bio101, Vista, CA), and ligated to form a heterogeneous population of a B7.1 expression vector, designated pTE3'B7.1. Without transforming *E. coli* and isolating individual clones, the entire population of ligated vector is linearized with *Xho* I and used as template for *in vitro* SP6 transcription reactions, as described above. The heterogeneous population of randomly mutagenized B7.1 vector transcripts is then electroporated into the desired cell type (for example, BHK cells) for initiation of the Sindbis replication cycle. Selection for virus mutants which establish a

25  
30



persistent infection or exhibit reduced, delayed, or no inhibition of host macromolecular synthesis is performed using a monoclonal antibody specific for B7.1 (Pharmingen, San Diego, CA) and either magnetic- or fluorescence-activated cell sorting protocols. The preferred secondary antibody tags include rat-anti-mouse IgG conjugated with magnetic  
5 microbeads for magnetic cell sorting (miniMACS Magnetic Separation System, Miltenyi Biotec, Auburn, CA; Miltenyi et al., *Cytometry* 11:231-238, 1990), and FITC-conjugated rat anti-mouse IgG (Pharmingen, San Diego, CA) for fluorescence activated cell sorting. Using such an approach and harvesting cells after an extended period (*see* above), only viable cells which contain a functional virus-derived vector (as evidenced  
10 by B7.1 expression), displaying the desired phenotype, are enriched.

Specifically, the heterogeneous population of randomly mutagenized B7.1 vector transcripts is electroporated into  $5 \times 10^7$  cells, according to the procedure of Liljestrom and Garoff (1991, *ibid*), and plated as a flat stock culture. At 12, 24, 36, and 48 hour post-infection, the cell monolayer is washed twice with fresh media to remove  
15 dead cells, and replaced with media consisting of a mixture of 50% fresh media and 50% conditioned media. After a desired time post-infection (for example 72 hours), the remaining cells are gently trypsinized to detach them from the culture dish, and pelleted by centrifugation at 1000 rpm, 4°C. The cells are resuspended in 2 ml of blocking solution (PBS + 10% fetal calf serum + 1% BSA), incubated on ice for 10 minutes, and  
20 re-pelleted. Next, the cells are resuspended in 200 ul of the primary anti-B7.1 antibody solution (diluted in PBS + 0.5% BSA), and incubated on ice for 30 minutes. The cells are washed twice with PBS + 0.5% BSA, pelleted, and resuspended in 200 ul of magnetic bead solution (200 ul washed magnetic rat anti-mouse coated beads in PBS + 0.5% BSA + 5 mM EDTA). Following incubation at 4°C for 30 minutes, the bead-  
25 bound cells are washed twice with PBS + 0.5% BSA + 5 mM EDTA, and resuspended in 1 ml of the same buffer. The bead-bound cells are then purified using the MiniMacs magnet column, according to the manufacturer's directions. The eluted positive cells are then re-seeded directly into a tissue culture dish containing a semi-confluent uninfected cell monolayer, at a ratio of 1 infected cell for every  $10^4$  uninfected cells.  
30 Additional rounds of selection are performed as above for amplification/enrichment.

The selected vector variants with the desired phenotype are isolated by limiting dilution or plaque purification, subjected to cDNA cloning, and the sequences responsible for the variant phenotype are isolated and characterized, as described previously.

Alternatively, the B7.1 expression vector, pTE3'B7.1, may be used  
5 directly for *in vitro* transcription without prior mutagenesis. Following transfection of these RNA transcripts, selection for mutants of the desired phenotype is performed as described above.

## 2. Vectors Expressing a Selectable Marker

10 Alternatively, an antibiotic resistance marker can be used for selection of virus vector variants exhibiting the desired phenotype. For example, the Sindbis vector pRSIN-luc (Dubensky *et al.*, *ibid*) is subjected to random mutagenesis as described above and a population of heterogeneous plasmid containing the random mutations is isolated. In addition, the gene encoding neomycin (G418) resistance is isolated by  
15 standard three-cycle PCR amplification, with 1.5 minutes extension, from plasmid pcDNA3 (Invitrogen, San Diego, CA), using the following oligonucleotide primers that are designed to contain flanking *Xho* I and *Not* I restriction sites:

Forward primer: NeoFX (5'-rest. site/neo sequence) (SEQ. ID. NO. 26)

20 5'-ATATACTCGAG/ACCAUGATTGAACAAGATGGATTG-3'

Reverse primer: NeoRN (5'-rest. site/neo sequence) (SEQ. ID. NO. 27)

5'-TATATAGCGGCCGC/TCAGAAGAACTCGTCAAGAAG-3'

25 Following amplification, the DNA fragment is purified with QIAquick-spin, digested with *Xho* I and *Not* I, and ligated into the mutated population of pRSIN-luc vector that also has been digested with *Xho* I and *Not* I, treated with calf intestinal alkaline phosphatase, and purified from a 0.7% agarose gel, away from its previous luciferase insert, using GENECLAN II. Ligation of the neomycin resistance marker  
30 into the mutated pRSIN vector creates a heterogeneous population of a neo' expression vectors, designated pRSIN-neo. Without transforming *E. coli* and isolating individual

clones, the entire population of ligated vector is linearized with *Sac I* and used as template for *in vitro* SP6 transcription reactions, as described above. The heterogeneous population of randomly mutagenized neo' vector transcripts is electroporated into the desired cell type (for example, BHK cells) for initiation of the

5 Sindbis replication cycle and heterologous gene expression. Approximately 12-24 hour post-transfection, the cells are trypsinized and replated in media containing G418. Subsequently, the media is changed at approximately 24 hour intervals to remove dead cells, and replaced with G418-containing media consisting of a mixture of 50% fresh media and 50% conditioned media. Media changes are reduced after the majority of

10 dead cells are washed away, and cell foci begin to form. Using this selection, only viable cells which contain a functional virus-derived vector, exhibiting the desired phenotype (as evidenced by neomycin resistance), are enriched. Mutant vector variant genomes displaying the desired phenotype are isolated by harvesting RNA directly from cell lysates using RNazol B (Tel-Test, Friendswood, TX). Alternatively, the cells are

15 transfected with a defective-helper Sindbis RNA (pR-dInsPSIN, Dubensky et al., *ibid*), which results in the production of packaged pRSIN-neo vector-containing particles in the culture supernatant, which may be concentrated by centrifugation for subsequent vector RNA isolation, as above. In each case, the vector RNA is subjected to cDNA cloning, and the sequences responsible for the variant phenotype are isolated and

20 characterized, as described previously.

Alternatively, the pRSIN vector with a neomycin resistance marker, may be used directly for *in vitro* transcription without prior mutagenesis. Following transfection of these RNA transcripts, selection for mutants of the desired phenotype is performed as described above.

25 In another example, a Semliki Forest virus derived vector pSFV-1 (GIBCO/BRL) is used for insertion of the antibiotic resistance marker and subsequent selection of the desired phenotype. The gene encoding neomycin (G418) resistance is isolated by standard three-cycle PCR amplification, with 1.5 minutes extension, from plasmid pcDNA3 (Invitrogen, San Diego, CA), using the following oligonucleotide

30 primers that are designed to contain flanking *BamH I* restriction sites:

Forward primer: 5'BAMHI-Neo (SEQ. ID. NO. 118)

5'-ATATAGGATCCTTCGCATGATTGAACAAGATGGATTGC-3'

Reverse primer: 3'BAMHI-Neo (SEQ. ID. NO. 57)

5'-ATATAGGATCCTCAGAAGAACTCGTCAAGAAGGCGA-3'

10                      Following amplification, the DNA fragment is purified with QIAquick-spin, digested with *BamH* I, and ligated into wild-type or a previously mutated population of pSFV-1 vector that also has been digested with *BamH* I, treated with calf intestinal alkaline phosphatase, and purified from a 0.7% agarose gel, using GENECLAN II. Transcription of RNA vectors and selection for mutants of the  
15      desired phenotype are carried out essentially as described above for Sindbis virus.

### EXAMPLE 3

#### PREPARATION OF SIN1-BASED RNA VECTOR REPLICONS

20

##### A.      Construction of the SIN-1 Basic Vector

            SIN-1 derived vector backbones were constructed and inserted into a plasmid DNA containing a bacteriophage RNA polymerase promoter, such that transcription *in vitro* produced an RNA molecule that acts as a self-replicating molecule  
25      (replicon) upon introduction into susceptible cells. The basic SIN-1 RNA vector replicon was comprised of the following ordered elements: SIN-1 nsPs genes, subgenomic RNA promoter region, a polylinker sequence, which may contain heterologous sequence insertions, the SIN-1 3' non translated region (NTR), and a polyadenylate sequence. Following transfection into susceptible cells, autonomous  
30      replication of the RNA vector replicon occurs as for virus, and the heterologous sequences are synthesized as highly abundant subgenomic mRNA molecules, which in turn serve as the translational template for the heterologous gene product.

The 5' region of the vector, comprised of the SIN-1 nsP genes and subgenomic promoter, extends to within two nucleotides of the capsid gene translational initiation point. This region was first inserted into the pKSII+ plasmid (Stratagene) between the *Apa* I and *Xho* I sites. The 5' region of the vector was amplified by PCR from the pRSIN-1g plasmid in two overlapping fragments. The first fragment was generated in a PCR reaction with the following primer pair:

Forward primer: SP6-1F(*Apa* I site/SP6 promoter/SIN nts 1-18): (SEQ. ID NO. 12)

5'-

10 TATATGGGCCCCGATTAGGTGACACTATAGATTGACGGCGTAGTACAC

Reverse primer: SIN5160R (SIN nts 5160-5140): (SEQ. ID NO. 28)

5'-CTGTAGATGGTGACGGTGTCG

15 The second fragment was generated in a PCR reaction with the following primer pair:

Forward primer: 5079F (SIN nts 5079-5100): (SEQ. ID NO. 29)

5'-GAAGTGCCAGAACAGCCTACCG

20 Reverse primer: SIN7643R (buffer sequence/*Xho* I site/ SIN nts 7643-7621):  
(SEQ. ID NO. 30)

5'-TATATCTCGAGGGTGGTGTGTAGTATTAGTCAG

The two PCR reactions were performed with the primer pairs shown above using the Thermalase (Amresco, Solon, OH), Vent (New England Biolabs, Beverly, MA) or KlenTaq thermostable DNA polymerases. Additionally, the reactions contained 5% DMSO and "HOT START WAX" beads (Perkin-Elmer, Foster City, CA). The PCR amplification protocol shown below was used. The extension period was 5 minutes or 2.5 minutes for reactions with the SP6-1F/SIN5160R or 30 5079F/SIN7643R primer pairs, respectively.

Temperature (°C)	Time (Min)	No. Cycles
95	2	1
95	0.5	35
55	0.5	
72	5.0 or 2.5	
72	10	10

Following PCR, the two amplified products of 5142 bp (SP6-1F/SIN5160R primer pair) and 2532 bp (5079F/SIN7643R primer pair) were purified (PCR purification kit, Qiagen, Chatsworth, CA), and digested with *Apa* I and *Sfi* I (5142 bp amplicon product) or *Sfi* I and *Xho* I (2532 bp amplicon product). The digested products were purified with GENECLAN II (Bio 101, Vista, CA) and ligated together with pKS+ plasmid (Stratagene, La Jolla, CA) prepared by digestion with *Apa* I and *Xho* I, and phosphatased with CIAP. This construction is known as pKSSIN-1-BV5'.

The 3' region of the vector, comprised of the viral 3' end, a polyadenylate tract, and a unique restriction recognition sequence were inserted between the *Not* I and *Sac* I sites of the plasmid pKSSIN-1-BV5'. The 3' region of the vector was amplified by PCR from the pRSIN-1g plasmid in a reaction containing the following primer pair:

Forward primer: SIN11386F (buffer sequence/*Not* I site/SIN nts 11386-11407):  
(SEQ. ID NO. 31)

5'-TATATATATATGCGGCCGCGCTACGCCCCAATGATCCGAC

Reverse primer: SIN11703R (buffer sequence/Sac I and Pme I sites/T40/SIN nts 11703-11698): (SEQ. ID NO. 32)

5'-CTATAGAGCT CGTTTAAACT TTTTTTTTTT TTTTTTTTTT  
TTTTTTTTTT TTTTTTTTTG AAATG

5

In addition to the primer pairs shown above, the PCR reaction contained Thermalase, Vent or KlenTaq thermostable DNA polymerase, 5% DMSO, and "Hot Start Wax" beads (Perkin-Elmer). The amplification protocol was as shown above, but with a 72°C extension period of 30 seconds.

10

The 377 bp amplified product corresponding to the 3' vector end was purified, digested with *Not* I and *Sac* I, purified, and ligated into pKSSIN-1-BV5', which was prepared by digestion with *Not* I and *Sac* I and treatment with CIAP. This plasmid is known as pKSSIN-1-BV.

15

Using techniques described above, the *lacZ* gene encoding the  $\beta$ -galactosidase reporter protein was liberated from the plasmid pSV- $\beta$ -galactosidase (Promega Corp., Madison, WI) with *Bam* HI and *Hind* III, and inserted into pKS+ at the corresponding enzyme recognition sites. The *lacZ* gene was digested from this plasmid, pKS- $\beta$ -gal, with *Xho* I and *Not* I, and inserted into pKSSIN-1-BV, between the *Xho* I and *Not* I sites. This plasmid is known as SINrep/SIN-1 nsP1-4/*lacZ*.

20

Alternatively, the firefly luciferase gene encoding the luciferase reporter protein was liberated from the plasmid pT3/T7-LUC (Clontech, Palo Alto, CA) by digestion with *Hind* III, and inserted into pKS+ (Stratagene, La Jolla, CA) at the corresponding enzyme recognition sites contained in the multiple cloning sequence to generate pKS-luc. The luciferase gene was liberated from pKS-luc by digestion with  
25 *Xho* I and *Not* I and inserted into *Xho* I/*Not* I digested pKSSIN-1-BV. This plasmid is known as SINrep/SIN-1 nsP1-4/*luc*.

Additionally, the gene encoding the secreted form of alkaline phosphatase (SEAP) was inserted into pKSSIN-1-BV. Briefly, the SEAP gene was liberated from the plasmid pCMV/SEAP (Tropix, Bedford, MA) by digestion with *Hind*  
30 III and *Xba* I, and inserted in pSK+ (Stratagene, La Jolla, CA) at the corresponding

recognition sites contained in the multiple cloning sequence, to generate pSK-SEAP. The SEAP gene was then liberated from pSK-SEAP by digestion with *Xho* I and *Not* I, and inserted into the corresponding enzyme recognition sites of pKSSIN-1-BV. This plasmid is known as SINrep/SIN-1 nsP1-4/SEAP.

5 The individual SIN-1 nsP genes were substituted into the corresponding wild-type virus region of the Sindbis virus-based lac Z replicon described previously (Bredenbeek et al., *J. Virol.* 67:6439-6446, 1993), in order to compare the expression properties of the SIN-1 and wild-type expression vectors. Substitution of the SIN-1 nsP genes into the Toto1101-derived lac Z replicon was accomplished as described in  
10 Example 1. These vectors were designated as shown in the table below.

Replicon Designation	nsP Genes Origin	
	Toto1101	SIN-1
SINrep/lacZ	nsP 1-4	
SINrep/SIN-1 nsP2/lacZ	nsP 1,3-4	nsP 2
SINrep/SIN-1 nsP3/lacZ	nsP 1-2, 4	nsP 3
SINrep/SIN-1 nsP1-4/lacZ		nsP 1-4

SP6 transcripts were prepared from the replicons shown in the table above, after linearization with *Xho* I, as described in Example 1. RNA transcripts  
15 contained a 5' sequence that is capable of initiating transcription of Sindbis virus, Sindbis virus nonstructural protein genes 1-4, RNA sequences required for packaging, a Sindbis virus junction region, the lacZ gene, and the Sindbis virus 3' end proximal sequences required for synthesis of the minus strand RNA.

The *in vitro* transcription reaction products or purified RNA were  
20 electroporated into baby hamster kidney-21 (BHK-21) cells as described previously (Liljestrom and Garoff, *Bio/Technology* 9:1356-1361, 1991). Alternatively, BHK-21 cells were complexed with a commercial cationic lipid compound as described in Example 1 and applied to BHK-21 cells maintained at 75% confluency. Transfected cells were propagated in 35 mm dishes, and incubated at 37°C.



The efficiency of transfection of BHK-21 cells with SINrep/lac Z RNAs after 9 hours was determined by two alternative methods. In the first method, transfected cells expressing  $\beta$ -galactosidase were determined by direct staining with X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), after first fixing cells with 2% cold methanol, as described previously (MacGregor et al., *Cell Mol. Genet.* 13:253-265, 1987). In the second method, expression of  $\beta$ -galactosidase were determined by immunofluorescence, using a rabbit anti- $\beta$ -galactosidase antibody. A portion of the cells transfected by either method described above were propagated on circular glass coverslips, contained in 35 mm dishes. At 9 hours post transfection (hpt), the media was removed by aspiration, and the cells were rinsed twice with PBS, and fixed with methanol by incubation overnight at -20°C. The methanol was removed by aspiration, the cells were rinsed three times with PBS, then incubated with 2% BSA (fraction V, Sigma, St. Louis, MO) for 30 minutes at room temperature. Following incubation with BSA to prevent non-specific antibody binding, the cells were incubated with the primary anti- $\beta$ -galactosidase antibody (diluted 1:800 in 0.1% BSA/PBS) for 1 hour at room temperature. Excess primary antibody was then removed by aspiration and rinsing three times with 0.1% BSA in PBS. Following 1:100 dilution in 2% BSA in PBS, 100 ml of goat anti-rabbit-FITC conjugate secondary antibody (Sigma, St. Louis, MO) were added to the coverslips and incubated for 45 minutes at room temperature, in the dark. Excess secondary antibody was removed by rinsing the coverslips twice with 0.1% BSA in PBS, and once with PBS. The coverslips were then mounted cell side down on a drop of Cytoseal 60 mounting media (Stephens Scientific, Riverdale, NJ), placed on a microscope slide. Fluorescence microscopy was used to determine the frequency of cells expressing  $\beta$ -galactosidase, in order to determine the transfection efficiency.

The level of  $\beta$ -galactosidase in whole transfected cell lysates was determined at 9 hpt, by two alternative methods. In the first method, transfected cells were rinsed with PBS after aspiration of the media, and 250  $\mu$ l of reporter lysis buffer (Promega, Madison, WI) per  $10^6$  cells was added to each dish.  $\beta$ -galactosidase expression levels were determined by mixing the supernatant fraction from cell lysates,

processed by micro centrifugation at 14,000 r.p.m. for 1 minute at room temperature, with a commercially available substrate detection system (Lumi-gal, Clontech, Palo Alto, CA), followed by luminometry (Analytical Luminescence Laboratory, San Diego, CA). In the second method, the activity of  $\beta$ -galactosidase was determined as described previously by Sambrook and Maniatis (1989, 2nd ed. Cold Spring Harbor Laboratory Press, NY). Briefly, transfected cells were rinsed with PBS after aspiration of the media, and 200  $\mu$ l of TTE lysis buffer (10 mM Tris-HCl pH 8.0/1 mM EDTA/0.2% Triton X-100) per  $10^6$  cells was added to each dish. Following pelleting of cell debris from the cell lysate by micro centrifugation at 14,000 r.p.m. for 1 minute at room temperature, 50  $\mu$ l of the supernatant was added to 0.5 ml of Z buffer pH 7.0 (60 mM  $\text{Na}_2\text{HPO}_4$ /40 mM  $\text{NaH}_2\text{PO}_4$ / 10 mM KCl/10 mM  $\text{MgSO}_4$ /50 mM 2-mercaptoethanol) and incubated at 37°C for 5 minutes.  $\beta$ -galactosidase activity in samples was then determined by spectrophotometry (420 nm) after addition of 0.2 ml of a solution containing 4 mg/ml of the chromogenic substrate o-nitrophenyl-b-D-galactopyranoside (ONPG; SIGMA, St. Louis, MO), in Z buffer. The samples were incubated at 37°C until the yellow color developed (approximately 5 minutes), and the reactions were terminated by addition of 0.5 ml of 0.5 M  $\text{Na}_2\text{CO}_3$ .

The level of  $\beta$ -galactosidase expression in transfected BHK-21 cells was determined according to the methods described above, and is illustrated in the table shown below. The data indicated are normalized for varying transfection efficiency, as described above.

Replicon Transfected	Expression of $\beta$ -gal, relative to SINrep/lacZ	Standard Deviation
SINrep/lacZ	1.0	
SINrep/SIN-1 nsP2/lacZ	3.2	$\pm 0.4$
SINrep/SIN-1 nsP3/lacZ	0.2	$\pm 0.0$
SINrep/SIN-1 nsP1-4/lacZ	5.2	$\pm 1.3$

The results demonstrate that the replicon vectors derived from the SIN-1 variant strain are indeed functional. When transfected into BHK-21 cells, the level of expressed reporter protein are higher than that observed in wild type virus-derived vector transfected cells. Furthermore, as with the phenotype for establishment of productive persistent infection, the higher level of  $\beta$ -galactosidase expression in BHK-21 cells transfected with the SIN-1-derived replicon vectors mapped primarily to the nsP 2 gene.

#### EXAMPLE 4

##### 10 PREPARATION OF SIN1-BASED DNA VECTORS

###### A. Construction of Plasmid DNA SIN-1 Derived Expression Vectors

Efficient initiation of the Sindbis virus infectious cycle can occur *in vivo* from a genomic cDNA clone contained within an RNA polymerase II expression cassette (Dubensky et al., *J. Virol* 70:508-519, 1996.). The ability to express functional alphavirus genes from a DNA format has enabled two new alphavirus-based gene expression systems to be developed: (1) a plasmid DNA-based vector with applications for genetic immunization, and (2) the production of packaged alphavirus particles in cells co-transfected with vector replicon and DH plasmid DNAs. Previously, molecular approaches to produce infectious Sindbis virus RNA and its derived complementary vectors were restricted primarily to *in vitro* transcription of cDNA clones from a bacteriophage RNA polymerase promoter followed by transfection into permissive cells.

The plasmid DNA-based alphavirus derived expression vector is known as ELVST<sup>TM</sup> (Eukaryotic Layered Vector System). The ELVST<sup>TM</sup> plasmid DNA vector involves the conversion of a self-replicating vector RNA (replicon) into a layered DNA-based expression system. Within certain embodiments the first layer has a eukaryotic (e.g. RNA polymerase II) expression cassette that initiates transcription of a second layer, which corresponds to the RNA vector replicon. Following transport of the replicon expressed from the first layer from the nucleus to the cytoplasm, autocatalytic

amplification of the vector proceeds according to the viral (*e.g.* alphavirus) replication cycle, resulting in expression of the heterologous gene.

Construction of SIN-1 virus-derived plasmid DNA expression vectors were performed with modifications of methods previously described (Dubensky et al., *J. Virol* 70:508-519, 1996). The expression vector was assembled on the plasmid vector pBGS131 (ATCC No. 37443), which is a kanamycin resistant analogue of pUC 9 (Spratt et al., *Gene* 41:337-342, 1986). pBGS131 and its derived plasmids were propagated in LB medium containing 20 µg/ml kanamycin.

To facilitate insertion of heterologous sequences into the expression vector, the *Xho* I recognition sequence, located within the translational reading frame of the kanamycin gene in pBGS131, was removed by inserting a partially complementary 12-mer oligonucleotide pair that contained *Xho* I sticky ends. The *Xho* I recognition site was lost as a result of the insertion, as shown below:

Oligonucleotide 1: (SEQ. ID NO. 33)

5'-TCGATCCTAGGA

Original pBGS131 sequence	Paired Oligonucleotides	pBGS131 sequence after insertion of 12-mer
SérArg		SerIleLeuGlySerArg
<u>CTCGAGGC</u>	TCGATCCTAGGA	CTCGATCCTAGGATCGAGGC
<u>GAGCTCCG</u>	AGGATCCTAGT	GAGCTAGGATCCTAGCTCCG

(*Xho* I site: CTCGAG) (SEQ. ID NOS. 33-36 and 104).

20

The oligonucleotide is gel annealed in equal molar concentrations in the presence of 10 mM MgCl<sub>2</sub>, heated to 100°C for 5 min, cooled slowly to room temperature, and phosphorylated with polynucleotide kinase. The oligonucleotide was ligated at a 200:1 ratio of insert:plasmid vector to pBGS131, which was prepared by *Xho* I digestion and CIAP treatment. The resulting plasmid is called pBGS131 dL*Xho* I.

25

The growth rates of XL1-Blue (Stratagene) transformed with pBGS131 or pBGS131 dL $\chi$ ho I plasmids in LB medium containing 20  $\mu$ g/ml kanamycin was indistinguishable over a time course between 1.5 and 8 hours.

The bovine growth hormone (BGH) transcription termination/polyadenylation signal was inserted between the *Sac* I and *Eco* RI sites of pBGS131 dL $\chi$ ho I. The BGH transcription termination sequences were isolated by PCR amplification using the primer pair shown below and the pCDNA3 plasmid (Invitrogen, San Diego, CA) as template.

10 Forward primer BGHTTF (buffer sequence/*Sac* I site/pCDNA3 nts 1132-1161):

(SEQ. ID NO. 37)

5'-TATATATGAGCTCTAATAAAATGAGGAAATTGCATCGCATTGTC

Reverse primer BGHTTR (buffer sequence/*Eco* RI site/pCDNA3 nts 1180-1154):

15 (SEQ. ID NO. 38)

5'-TATATGAATTCATAGAATGACACCTACTCAGACAATGCGATGC

The primers shown above were used in a PCR reaction with a three temperature cycling program, using a 30 sec extension period. The 58 bp amplified product was purified with the PCR purification kit (Qiagen Chatsworth, CA), digested with *Sac* I and *Eco* RI, purified with GENE CLEAN II, and ligated into *Sac* I/*Eco* RI digested, CIAP treated pBGS131. The plasmid is known as pBGS131 dL $\chi$ ho I-BGHTT.

The 3' end of the Sindbis virus-derived plasmid DNA expression vector was then inserted into the pBGS131 dL $\chi$ ho I-BGHTT construct. This region of the vector contains the following ordered elements: Sindbis virus 3' end non-translated region (3' NTR); a 40-mer poly(A) sequence; the hepatitis delta virus (HDV) antigenomic ribozyme; and a *Sac* I recognition sequence. Construction of these ordered elements was accomplished by nested PCR, using the primers shown below and the pKSSIN-1-BV plasmid (Example 4) as template.

Forward primer: SIN11386F (buffer sequence/Not I site/SIN nts 11386-11407):  
(SEQ. ID NO. 31)

5'-TATATATATATGCGGCCGCGCTACGCCCCAATGATCCGAC

5 Nested primer: pAHDV1F (poly(A)/HDV RBZ nts 1-46): (SEQ. ID NO. 39)

5'-AAAAAAAAAA GGGTCGGCAT GGCATCTCCA CTCCTCGCG  
GTCCGACCTG GGCATC

Reverse primer: SacHDV77R (buffer sequence/Sac I site/HDV RBZ nts 77-27):

10 (SEQ. ID NO. 40)

5'-TATATGAGCTCCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTT  
CGGATGCCCAGGTCGGACCGCG

The primers shown above were used in a PCR amplification according to  
15 the reaction conditions and three temperature cycling program described in Example 4,  
with an extension time of 30 sec. The 422 bp amplified product was purified with a  
PCR purification kit (Qiagen Chatsworth, CA), digested with *Not* I and *Sac* I, purified  
with GENECLAN II, and ligated into *Not* I/*Sac* I digested, CIAP-treated pKSSIN-1-  
BV. This construct is known as pKSSIN-1BV/HDVRBZ and contains Sindbis virus-  
20 derived plasmid DNA expression vector sequences from the *Bgl* II site at Sindbis nt  
2289 extending through the 3' end of the vector including the HDV ribozyme sequence.

Plasmid pKSSIN-1BV/HDVRBZ was then digested with *Bgl* II and  
*Sac* I, the 5815 bp fragment was isolated by 1% agarose/TBE gel electrophoresis,  
purified with GENECLAN II, and was inserted into *Bgl* II/*Sac* I digested, CIAP-  
25 treated pBGS131 dLXho I-BGHTT to generate the plasmid construct known as  
pBG/SIN-1BglLF. This construct contains the region of the Sindbis virus expression  
vector from plasmid pKSSIN-1BV/HDVRBZ described above with the 3' end fused to  
the BGH transcription termination sequence on the pBGS131 dLXho I plasmid.

Assembly of the Sindbis virus plasmid DNA vector was completed by  
30 insertion of the CMV promoter juxtaposed with the first 2289 nts of the Sindbis virus

genome (includes the 5' viral end and a portion of the nsPs genes) into the pBG/SIN-1BglLF plasmid. Using an overlapping PCR approach, the CMV promoter was positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the 5' end of the Sindbis virus vector replicon RNA. The

5 CMV promoter was amplified in a first PCR reaction from pCDNA3 (Invitrogen, San Diego, CA) using the following primer pair:

Forward primer: pCBgl/233F (buffer sequence/Bgl II recognition sequence/CMV promoter nts 1-22): (SEQ. ID NO. 41)

10 5'-TATATATAGATCTTTGACATTGATTATTGACTAG

Reverse primer: SNCMV1142R (SIN nts 8-1/CMV pro nts 1142-1108):  
(SEQ. ID NO. 42)

15 5'-CCGTCAATACGGTTCCTAAACGAGCTCTGCTTATATAGACC

The primers shown above were used in a PCR reaction according to the reaction conditions and three temperature cycling program described in Example 4, with an extension time of 1 min.

The SIN-1 5' end was amplified in a second PCR reaction from

20 pKRSIN-1g clone (Example 1) using the following primer pair:

Forward primer: CMVSIN1F (CMV pro nts 1124-1142/SIN nts 1-20):  
(SEQ. ID NO. 43)

25 5'-GCTCGTTTAGTGAACCGTATTGACGGCGTAGTACACAC

Reverse primer: SIN 3182R (SIN nts 3182-3160): (SEQ. ID NO. 44)

5'-CTGGCAACCGGTAAGTACGATAC

5 The primers shown above were used in a PCR reaction with a three temperature cycling program using a 3 min extension period.

The 930 bp and 3200 bp amplified products were purified with a PCR purification kit (Qiagen) and used together in a PCR reaction with the following primer pair:

10

Forward primer: pCBgl233F: (SEQ. ID NO. 41)

5'-TATATATAGATCTTTGACATTGATTATTGACTAG

15 Reverse primer: (SIN nts 2300-2278): (SEQ. ID NO. 45)

5'-GGTAACAAGATCTCGTGCCGTG

20 The primers shown above were used in a PCR reaction with a three temperature cycling program using a 3.5 min extension period.

The 26 3' terminal bases of the first PCR amplified product overlap with the 26 5' terminal bases of the second PCR amplified product; the resultant 3200 bp overlapping secondary PCR amplified product was purified by 1% agarose/TBE electrophoresis, digested with *Bgl* II, and ligated into *Bgl* II digested, CIAP-treated  
25 pBG/SIN-1BglLF. This construct is called pBG/SIN-1 ELVS 1.5.

As discussed within Example 1, relatively few nucleotide point changes in the nsP gene sequence of wild-type Sindbis virus result in the phenotype characteristic of SIN-1. No new restriction enzyme recognition sites are generated as a result of these nucleotide changes which facilitate clones derived from wild-type and  
30 SIN-1 genotypes to be easily distinguished. A PCR-based diagnostic assay was



therefore devised as a rapid method for identification of SIN-1 derived clones. Briefly, forward primers were designed so that a particular base change between SIN-1 and wild-type was positioned at the 3' terminal base of the primer. One primer contained the SIN-1 nucleotide while another contained the wild-type nucleotide. A reverse primer in a region downstream conserved between both genotypes was used in combination with each forward primer. At the correct annealing temperature, SIN-1 templates were only amplified in reactions containing SIN-1 forward primers. The primer sequences used to distinguish wild-type and SIN-1 genotypes is given below. The reaction conditions were as described throughout the examples contained herein.

10

Primer Set 1:

Forward primers:

WT100F                    5'-GTC CGT TTG TCG TGC AAC TGC  
(SEQ. ID NO. 105)

15

SIN-1100F: 5'-GTC CGT TTG TCG TGC AAC TGA  
(SEQ. ID NO. 106)

Reverse primer:

20

SIN2300R

PCR Program:            (95°C-30", 72°C-2') 20 cycles

Primer Set 2:

25

Forward primers:

WT3524F                    5'-CAA TCT TCC TCA CGC CTT AGC  
(SEQ. ID NO. 107)

30

SIN-13524F 5'-CAA TCT TCC TCA CGC CTT AGT  
(SEQ. ID NO. 108)

Reverse primer:

SIN5448R

5 PCR Program: (95°C-30", 60°C-30", 72°C-2') 20 cycles

Primer Set 3:

Forward primers:

WT7592F 5'TCC TAA ATA GTC AGC ATA GTA

10 (SEQ. ID NO. 109)

SIN-17592F 5'TCC TAA ATA GTC AGC ATA GTT

(SEQ. ID NO. 110)

15 Reverse primer:

SIN7643R 5'-TATATCTCGAGGGTGGTGTGTTGTAGTATTAGTCAG

(SEQ. ID NO. 111)

PCR Program: (95°C-30", 60°C-30", 72°C-2') 20 cycles

20

Reporter protein expression vectors were constructed by inserting the lacZ, SEAP, or luciferase reporter genes into the pBG/SIN-1 ELVS 1.5 vector backbone. In separate reactions, the pKS-β-gal, pSK-SEAP, and pKS-luc plasmids (Example 4), were digested with *Xho* I and *Not* I. The fragments containing the lacZ, SEAP, or luciferase genes were isolated by 1% agarose/TBE gel electrophoresis and purified subsequently with GENECLAN II. These reporter genes were then ligated in separate reactions with *Xho* I/*Not* I digested, CIAP-treated pBG/SIN-1 ELVS 1.5 plasmid. These constructs are known as pBG/SIN-1 ELVS 1.5-β-gal, pBG/SIN-1 ELVS 1.5-SEAP, and pBG/SIN-1 ELVS 1.5-luc.

30

B. Expression of Heterologous Proteins in Cells Transfected with pBG/SIN-1 ELVS 1.5-SEAP, pBG/SIN-1 ELVS 1.5-luc or pBG/SIN-1 ELVS 1.5- $\beta$ -gal Expression Vectors

The pattern of secreted alkaline phosphatase, luciferase, and  $\beta$ -galactosidase reporter gene expression in BHK cells transfected with pBG/SIN-1 ELVS 1.5 or pBG/wt ELVS 1.5 vectors was compared. The pBG/wt ELVS 1.5 plasmid contains sequences derived from wild-type Sindbis virus, rather than the SIN-1 variant. Construction of the pBG/wt ELVS 1.5 expression vectors was exactly as described herein for the pBG/SIN-1 ELVS 1.5 expression vectors, except that full-length genomic cDNA derived from wild-type Sindbis virus (Dubensky et al., WO 95/07994) was used as the template for the vector construction. Construction of the pBG/wt ELVS 1.5 expression vector has been described previously (Dubensky, *supra.*); thus, although the strains, and therefore the sequences, are different, the Sindbis virus-specific regions contained in the pBG/wt ELVS 1.5 and pBG/SIN-1 ELVS 1.5 expression vectors are the same.

Baby hamster kidney-21 (BHK-21) cells maintained at 75% confluency in 12 mm dishes were transfected with 1.0  $\mu$ g of pBG/SIN-1 ELVS 1.5 or pBG/wt ELVS 1.5 expression vector plasmid DNAs complexed with 4.0  $\mu$ l of a commercially available lipid (Lipofectamine, GIBCO-BRL). Otherwise, transfection conditions were as suggested by the lipid manufacturer. Eagle minimal essential medium supplemented with 5% fetal bovine sera was added to the cells at 4 hours post transfection (hpt), unless otherwise indicated. Transfected cells were incubated at 37°C. At various times post transfection, as indicated below, several assays were performed to compare vector-specific RNA synthesis, and expression of secreted alkaline phosphatase, luciferase, or  $\beta$ -galactosidase reporter gene expression in cells transfected with pBG/SIN-1 ELVS 1.5 or pBG/wt ELVS 1.5 plasmid DNAs.

The levels of alkaline phosphatase secreted into the culture medium of BHK cells transfected with pBG/SIN-1 ELVS-1 1.5-SEAP or pBG/wt ELVS 1.5-SEAP plasmid DNA were compared. Cell culture medium was assayed for the presence of alkaline phosphatase with the Phospha-Light™ chemiluminescent reporter gene assay, according to the directions of the manufacturer (Tropix, Inc., Bedford, MA). Briefly,

10  $\mu$ l of cell culture supernatant was mixed with 30  $\mu$ l of Dilution Buffer and incubated for 30 minutes at 65°C. The sample was allowed to cool to room temperature before mixing with 40  $\mu$ l of Assay Buffer. The sample was incubated for five minutes at room temperature followed by the addition of 40  $\mu$ l of Reaction Buffer. Samples were  
 5 incubated for 20 minutes at room temperature. Total luminescence was measured on an ML3000 microtiter plate luminometer (Dynatech, Inc., Chantilly, VA) in cycle mode. and alkaline phosphatase (AP) in the culture medium of BHK cells transfected with pBG/SIN-1 ELVS-1 1.5-SEAP or pBG/wt ELVS 1.5-SEAP plasmid DNAs, were determined at 48 hpt, and the results are shown in the table below.

10

Plasmid Transfected	RLU at 48 hpt
pBG/SIN-1 ELVS 1.5-SEAP	18 $\pm$ 1.7
pBG/wt ELVS 1.5-SEAP	94 $\pm$ 10.7
pCDNA3	0.13 $\pm$ 0.04

Additionally, the levels of vector-specific RNAs synthesized in BHK-21 cells transfected with pBG/SIN-1 ELVS 1.5-SEAP or pBG/wt ELVS 1.5-SEAP plasmids were determined by Northern blot analysis, exactly as described previously  
 15 (Dubensky, *supra.*), at 48 hours post-transfection. The results of this experiment are shown in Figure 9A. Total cellular RNA was isolated from transfected BHK cells with Tri-Reagent as described by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH). Total cellular RNA concentrations present in samples from transfected BHK cells were determined spectrophotometrically. Additionally, material  
 20 isolated from transfected cells was determined to be intact by electrophoresis of 0.5  $\mu$ g of total cellular RNA through 0.7% agarose/TBE mini gels, stained 10  $\mu$ l/ml of ethidium bromide. Northern blot analysis was performed according to Sambrook and Maniatis (1989, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). In order that RNA from all transfected samples could be visualized on a autoradiogram  
 25 from a single Northern blot analysis, 2.5  $\mu$ g and 30  $\mu$ g of RNA were loaded per lane from pBG/wt ELVS 1.5-SEAP and pBG/SIN-1 ELVS 1.5-SEAP transfected cells,

respectively. Four samples of RNA, from individual transfections with both plasmids tested, were electrophoresed through 0.7% formaldehyde agarose gels and transferred to Zeta-probe membrane (Bio-Rad, Richmond, CA). The blot was hybridized with random-primed probes corresponding to the alkaline phosphatase gene. The results of this experiment in which the levels of vector-specific RNA synthesis and AP expression in transfected BHK cells were compared at 48 hpt, demonstrate that while the level of vector-specific RNA synthesized in cells transfected with pBG/SIN-1 ELVS 1.5-SEAP DNA was at least 100-fold lower than in pBG/wt ELVS 1.5-SEAP transfected cells, the levels of AP were only 5-fold lower in cells transfected with pBG/SIN-1 ELVS 1.5-SEAP DNA, compared to pBG/wt ELVS 1.5-SEAP DNA.

The levels of alkaline phosphatase secreted into the culture medium of BHK cells transfected with pBG/SIN-1 ELVS-1 1.5-SEAP or pBG/wt ELVS 1.5-SEAP plasmid DNA were also compared over a 7 day time-course. The results of this study, illustrated in Figure 9B, demonstrate that the levels of AP present in the culture medium at early time points were much lower in cells transfected with pBG/SIN-1 ELVS-1 1.5-SEAP plasmid, compared to pBG/wt ELVS 1.5-SEAP plasmid. However, the level of AP expressed in cells transfected with the SIN-1 Sindbis virus variant strain-derived vectors rapidly increased and was higher than in cells transfected with wild-type virus-derived vectors by the 96 hpt time point.

The luciferase levels present in BHK cells transfected with pBG/SIN-1 ELVS-1 1.5-luc or pBG/wt ELVS 1.5-luc plasmid DNA were compared at 24, 48, and 72 hpt. The luciferase expression levels were quantitated by adding 250  $\mu$ l of reporter lysis buffer (Promega, Madison WI) per  $10^6$  transfected cells, centrifuging the lysate at 14,000 rpm for 1 min, and then mixing the supernatant fraction from the cell lysates with a commercially available substrate detection system (Promega, Madison WI), followed by luminometry (Analytical Luminescence Laboratory, San Diego, CA). The results from this experiment (shown in the table below), and shown graphically in Figure 10, parallel the results observed with the alkaline phosphatase expression vectors. At early times post transfection the luciferase expression levels were lower in BHK cells transfected with pBG/SIN-1 ELVS 1.5-luc plasmid, compared to pBG/wt

ELVS 1.5-luc plasmid. However, at the 48 and 72 hpt time points, the luciferase levels were similar in BHK cells transfected with Sindbis virus SIN-1 variant strain- and wild-type-derived expression vectors.

Plasmid Transfected	Hr. Post Transfection	Relative Light Units (Ave, $\pm$ SD)
pBG/SIN-1 ELVS 1.5-luc	24	$1.2 \times 10^9$
pBG/wt ELVS 1.5-luc		$2.1 \times 10^8$
pBG/SIN-1 ELVS 1.5-luc	48	$3.3 \times 10^9$
pBG/wt ELVS 1.5-luc		$4.1 \times 10^9$
pBG/SIN-1 ELVS 1.5-luc	72	$1.8 \times 10^9$
pBG/wt ELVS 1.5-luc		$5.8 \times 10^9$
pCDNA3	48	482

5

The efficiency of transfection of the pBG/SIN-1 ELVS 1.5- $\beta$ -gal and pBG/wt ELVS 1.5- $\beta$ -gal plasmids in BHK cells at 48 hpt was determined by direct X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) staining of the cell monolayer (MacGregor, *Cell Mol. Genet.* 13:253-265, 1987), in order to measure directly the number of cells expressing  $\beta$ -galactosidase. The transfection efficiencies were equivalent, and are shown in the table below.

10

Plasmid Transfected	No. Blue Cells/100X Field
pBG/SIN-1 ELVS 1.5- $\beta$ -gal	$24 \pm 3$
pBG/wt ELVS 1.5- $\beta$ -gal	$26 \pm 7$

The levels of vector-specific RNAs synthesized in BHK-21 cells transfected with pBG/SIN-1 ELVS 1.5- $\beta$ -gal or pBG/wt ELVS 1.5- $\beta$ -gal plasmids

15

were determined by Northern blot analysis, exactly as described previously (Dubensky, *supra.*), at 48 and 72 hours post-transfection. Total cellular RNA was isolated from transfected BHK cells with Tri-Reagent as described by the manufacturer (Molecular Research Center, Inc., Cincinnati, OH). Total cellular RNA concentrations present in  
5 samples from BHK cells transfected with pBG/SIN-1 ELVS 1.5- $\beta$ -gal or pBG/wt ELVS 1.5- $\beta$ -gal plasmids were determined spectrophotometrically. Additionally, material isolated from transfected cells was determined to be intact by electrophoresis of 0.5  $\mu$ g of total cellular RNA through 0.7% agarose/TBE mini gels, stained 10  $\mu$ l/ml of ethidium bromide. Northern blot analysis was performed according to Sambrook and  
10 Maniatis (1989, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). In order that RNA from all transfected samples could be visualized on a autoradiogram from a single Northern blot analysis, 2.5  $\mu$ g and 5  $\mu$ g of RNA were loaded per lane from pBG/wt ELVS 1.5- $\beta$ -gal and pBG/SIN-1 ELVS 1.5- $\beta$ -gal transfected cells, respectively. Two samples of RNA, from individual transfections with both plasmids  
15 tested, at 48 and 72 hpt, were electrophoresed through 0.7% formaldehyde agarose gels and transferred to Zeta-probe membrane (Bio-Rad, Richmond, CA). The blot was hybridized with random-primed probes corresponding to the  $\beta$ -galactosidase gene. The results of this experiment, shown in Figure 11A, demonstrate that the level of vector specific RNA synthesized in cells transfected with pBG/SIN-1 ELVS 1.5- $\beta$ -gal DNA at  
20 48 and 72 hours post transfection was at least 100-fold lower than the level of RNA detected in pBG/wt ELVS 1.5- $\beta$ -gal transfected cells.

Additionally the  $\beta$ -galactosidase expression levels were quantitated in transfected whole cell lysates by adding 250  $\mu$ l of reporter lysis buffer (Promega, Madison WI) per  $10^6$  transfected cells, centrifuging the lysate at 14,000 rpm for 1 min,  
25 and then mixing the supernatant fraction from the cell lysates with a commercially available substrate detection system (Clontech, Palo Alto, CA), followed by luminometry (Analytical Luminescence Laboratory, San Diego, CA). The results from this experiment (shown in the table below, and graphically in Figure 9C) demonstrate that at early times post transfection the  $\beta$ -galactosidase expression levels were  
30 significantly lower in BHK cells transfected with pBG/SIN-1 ELVS 1.5- $\beta$ -gal plasmid,

compared to pBG/wt ELVS 1.5- $\beta$ -gal plasmid. However, reporter expression rapidly increased over the time-course in pBG/SIN-1 ELVS 1.5- $\beta$ -gal transfected cells such that the  $\beta$ -galactosidase levels were higher than in wild-type virus transfected cells at the final 120 hr time point.

5

Plasmid Transfected	Hr. Post Transfection	Relative Light Units (Ave, $\pm$ SD)
pBG/SIN-1 ELVS 1.5- $\beta$ -gal	24	54030 $\pm$ 7348
pBG/wt ELVS 1.5- $\beta$ -gal		4801590 $\pm$ 74,425
pBG/SIN-1 ELVS 1.5- $\beta$ -gal	48	310830 $\pm$ 31083
pBG/wt ELVS 1.5- $\beta$ -gal		2214921 $\pm$ 248071
pBG/SIN-1 ELVS 1.5- $\beta$ -gal	72	1443474 $\pm$ 98156
pBG/wt ELVS 1.5- $\beta$ -gal		3793524 $\pm$ 857336
pBG/SIN-1 ELVS 1.5- $\beta$ -gal	96	2232585 $\pm$ 299166
pBG/wt ELVS 1.5- $\beta$ -gal		3514262 $\pm$ 548225
pBG/SIN-1 ELVS 1.5- $\beta$ -gal	120	3200910 $\pm$ 128036
pBG/wt ELVS 1.5- $\beta$ -gal		1986537 $\pm$ 166869
pCDNA3		3637

Expression of  $\beta$ -galactosidase in cells transfected with the pBG/SIN-1 ELVS-1 1.5- $\beta$ -gal or pBG/wt ELVS 1.5- $\beta$ -gal plasmid DNAs was also measured directly by Western blot analysis using a monoclonal antibody specific for the reporter protein (Boehringer Mannheim), at the final 120 hpt time point. In parallel with the reporter protein activity determined at 120 hpt, the level of  $\beta$ -galactosidase protein was

10



at the same level, or greater, in PBG/SIN-1 ELVS-1 1.5- $\beta$ -gal transfected BHK cells, compared to pBG/wt ELVS 1.5- $\beta$ -gal, and is demonstrated in Figure 11C.

Taken together, the results described herein demonstrate that the level of vector-specific RNA synthesized is at least 100-fold less in pBG/SIN-1 ELVS  
5 transfected cells, compared to pBG/wt ELVS transfected cells. Importantly however, after a 48-72 hour lag, the levels of reporter gene expression are equivalent, or higher, in pBG/SIN-1 ELVS transfected cells, compared to pBG/wt ELVS transfected cells. The phenotype of pBG/SIN-1 ELVS, characterized by high expression levels combined with low vector-specific RNA synthesis in transfected cells, is due likely to the  
10 diminished, or absent, inhibition of host cell protein synthesis. This property of pBG/SIN-1 ELVS thus results in much higher levels of expressed reporter protein per subgenomic mRNA translation template in transfected cells, compared to pBG/wt ELVS. In summary, the phenotype of the plasmid DNA expression vectors derived from the SIN-1 variant strains follows the parent virus, in terms of equivalent  
15 expression levels, combined with relatively low levels of RNA synthesis, compared to wild-type virus derived-vectors. As vectors do not contain any of the Sindbis virus structural proteins, this phenotype must map to the nonstructural genes of the SIN-1 virus variant.

20

#### EXAMPLE 5

##### MODIFICATIONS OF PLASMID DNA SIN-1 DERIVED EXPRESSION VECTORS

Expression levels of heterologous genes in target cells from alphavirus-  
25 based vectors are effected by several factors, including host genus and vector configuration. For example,  $\beta$ -galactosidase expression levels are 10- to 100-fold higher in BHK cells, compared to some human cells, such as HT1080, transfected with pBG/ELVS vectors. The levels of reporter gene expression in BHK and several human cell lines transfected with pBG/wt ELVS 1.5- $\beta$ gal plasmid DNA (see example 4) were  
30 compared in order to establish the relative level of vector-specific expression in cell

types derived from the intended *in vivo* target genus. The levels of  $\beta$ -galactosidase expression in BHK cells and HT1080 (ATCC CCL 121) cells, a human fibrosarcoma line, transfected with pBG/wt ELVS 1.5- $\beta$ gal plasmid, or with a conventional plasmid expression vector, were determined. The conventional plasmid vector was constructed  
5 by insertion of the lac Z gene (Promega, Madison, WI) into the CMV promoter-driven pUC-derived expression plasmid multiple cloning site (Invitrogen, San Diego, CA), and is known as pCMV- $\beta$ -gal. The results of this study, given in Figure 12A, demonstrate that the level of  $\beta$ -galactosidase expression was nearly 100-fold lower in HT1080 cells transfected with pBG/wt ELVS 1.5- $\beta$ gal plasmid DNA, compared to BHK cells. Cells  
10 were also transfected with pCMV- $\beta$ -gal in order to segregate RNA polymerase II expression from Sindbis virus vector replicon expression. In this experiment, while the expression decreased 5- to 10-fold in HT1080 cells transfected with pCMV- $\beta$ -gal plasmid, compared to BHK cells, expression decreased nearly 100-fold in HT 1080 cells transfected with pBG/wt ELVS 1.5- $\beta$ gal plasmid DNA. Thus, the results indicate  
15 that the dramatic decrease of reporter gene expression in HT1080 cells transfected with pBG/wt ELVS 1.5- $\beta$ gal plasmid DNA is due in part to the diminished activity of the Sindbis virus vector replicon in these human cells.

Given the overall plasticity of the RNA alphaviral genome and the propagation of virus in BHK cells, it is not surprising that the expression levels of  
20 heterologous genes are highest in the host cell lines from which the vectors were derived. Thus, selection of alphaviruses with the SIN-1 phenotype (as described in Examples 1 and 2), characterized by comparatively low viral RNA levels and equivalent virus production levels, combined with delayed or absent inhibition of host cell protein synthesis, can be performed in any human primary, or diploid or polyploid human cells.

25 In addition to selecting alphaviruses with desired phenotypes in cells (e.g., human) which more closely parallel target cells *in vivo*, several alternative modifications of the prototype plasmid DNA expression cassette components can also be performed. For example, substitution of the MoMLV RNA polymerase II promoter with the stronger CMV immediate early (IE) promoter significantly enhances the level  
30 of heterologous gene expression in transfected cells (Dubensky et al., *J. Virol.* 70:508-

519, 1996, and Dubensky et al., W/O 95/07994). Further, juxtaposition of introns, for example SV40 small t antigen or CMV intron A, either upstream or downstream from the heterologous gene, can increase the level of heterologous gene expression in some transfected cell types (Dubensky et al., *supra.*).

5               Several further alternative modifications of the prototype plasmid DNA expression cassette components can also be utilized in order to enhance the overall expression in transfected cells *in vitro* or *in vivo*. In one modification, the Hepatitis B virus (HBV) posttranscriptional regulatory element (PRE) was inserted in the pBG/wt ELVS 1.5- $\beta$ gal plasmid DNA. The PRE sequence activates the transport of HBV S  
10 transcripts in *cis* from the nucleus to the cytoplasm. The PRE sequence appears to function independently of splice donor and acceptor sites, and has been shown to activate cytoplasmic expression of a  $\beta$ -globin transcript not containing introns. It has been proposed that the PRE functions in *cis* to allow the export of nuclear transcripts that do not interact efficiently with the splicing pathway and hence are not exported  
15 well from the nucleus (Huang et al., *Molecular and Cellular Biology* 15:3864-3869, 1995).

The PRE sequence was cloned into pBG/SIN-1 ELVS 1.5- $\beta$ gal by isolating first a PCR-generated 564 bp fragment of HBV from the full length genomic clone of the ADW viral strain, pAM6 (ATCC No. 39630). The amplified fragment  
20 extends from base 1238-1802 of the HBV genome. The primer sequences are given below.

Forward Primer: NTPRE1238F (SEQ. ID NO. 46)

5'-CCTATGCGGCCGCGTGGAACCTTTGTGGCTCCTC

25

Reverse Primer: EAPRE1802R (SEQ. ID NO. 47)

5'-CCTATTGGCCAGCAGACCAATTTATGCCTAC

30

The primers introduce a *Not* I recognition site at the 5' end of the fragment and an *Eae* I recognition site at the 3' end. *Not* I and *Eae* I have compatible sticky ends. The *Eae* I recognition site is internal to the *Not* I site, so *Eae* I cuts both sites.

5           The PCR fragment was digested with *Eae* I and cloned into *Not* I digested/CIAF treated pBG/wt ELVS 1.5- $\beta$ gal. The correct clone retains the *Not* I site at the 5' terminus of the PRE and is called pBG/wt ELVS/PRE 1.5- $\beta$ gal.

          The possible effect of the PRE sequence contained in ELVS plasmids on heterologous gene expression in transfected cells was determined. Briefly, BHK and  
10 HT1080 cells were cultured in 12 mm dishes to 75% confluency and transfected with 500 ng of pCMV- $\beta$ -gal, pBG/wt ELVS 1.5- $\beta$ gal, or pBG/wt ELVS/PRE 1.5- $\beta$ gal plasmid DNA complexed with Lipofectamine (GIBCO-BRL, Gaithersburg, MD), and the level of  $\beta$ -galactosidase expression was determined 48 hr later. Transfection efficiencies were determined by direct Xgal staining of transfected monolayers, as  
15 described in Example 4, and are shown in the table below.

<u>Construct</u>	<u>No. Blue cells/12 mm Dish</u>	
	<u>BHK</u>	<u>HT1080</u>
pCMV- $\beta$ -gal	549	42
pBG/wt ELVS 1.5- $\beta$ gal	146	1
pBG/wt ELVS/PRE 1.5- $\beta$ gal	334	33
Mock	0	0

The results demonstrate clearly that the number of BHK or HT1080 cells transfected with ELVS plasmids expressing  $\beta$ -galactosidase was increased dramatically by inclusion of the RNA transport PRE sequence in the vector. Further, these results  
20 indicate that one cause for the diminished heterologous gene expression levels in HT1080 cells, compared to BHK cells, transfected with ELVS plasmid DNA is the inefficient transport of the primary transcript from the nucleus.

          In parallel with the higher frequency of reporter protein expressing HT1080 cells transfected with ELVS plasmids containing the PRE sequence, the levels  
25 of  $\beta$ -galactosidase were dramatically higher in lysates from HT1080 cells transfected

with ELVS vectors containing the PRE sequence. These results are illustrated in Figure 12B, and taken together with the results shown in the table above, demonstrate that functional vector replicons are transported inefficiently from the nucleus in human cells transfected with ELVS plasmids. Further, inclusion of the PRE sequence in the ELVS plasmid construct increases the level of heterologous gene expression in all cells tested, demonstrating a clear relationship between efficiency of cytoplasmic vector replicon transport and overall heterologous gene expression level.

Several other viral sequence elements which operate in *cis* to transport unspliced RNAs have also been identified. For example, a 219 bp sequence, located between nts 8022 and 8240 near the 3' end of the Mason-Pfizer monkey virus (MPMV) genome, has been shown to enable Rev independent human immunodeficiency virus type 1 (HIV-1) replication (Bray et al., *PNAS* 91:1256-1260, 1994). The MPMV RNA transport element, known as the constitutive transport element (CTE), is inserted into the pBG/SIN-1 ELVS 1.5- $\beta$ gal plasmid by first isolating a PCR-generated 219 bp fragment of MPSV from the full length genomic clone template (Sonigo et al., *Cell* 45:375-385, 1986), or the MPSV subgenomic clone pGEM7FZ(-)MPSV 8007-8240 (D. Rekosh, Ham-Rek Laboratories, SUNY at Buffalo, 304 Foster Hall, Buffalo, New York). The amplified fragment extends from base 8022-8240 of the MPSV genome. The primer sequences are given below.

20

Forward Primer: NMPVM8021F (SEQ. ID NO. 48)

5'-CCTATGCGGCCGCTAGACTGGACAGCCAATGACG

25 Reverse Primer: EMPMV8241R (SEQ. ID NO. 49)

5'-CCTATTGGCCAGCCAAGACATCATCCGGGCAG

The primers introduce a *Not* I recognition site at the 5' end of the fragment and an *Eae* I recognition site at the 3' end. *Not* I and *Eae* I have compatible

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sticky ends. The *Eae* I recognition site is internal to the *Not* I site, so *Eae* I cuts both sites.

The PCR fragment is digested with *Eae* I and cloned into *Not* I digested/CIAP treated pBG/wt ELVS 1.5- $\beta$ gal. The correct clone retains the *Not* I site  
5 at the 5' terminus of the PRE and is called pBG/wt ELVS/CTE 1.5- $\beta$ gal.

In addition to the HBV PRE and MPSV CTE sequences, several RNA transport elements from other viral or cellular sources can be inserted into the ELVS plasmid constructs, as described above. For example, some of these elements include the HIV Rev responsive element (Malim et al., *Nature*, 338:254-257, 1989), the HTLV  
10 1 Rex element (Ahmed et. al, *Genes Dev.*, 4:1014-1022, 1990), and another *cis*-acting sequence from simian retrovirus type 1 (Zolotukhin et al., *J. Virol.*, 68:7944-7952, 1994). In addition, each of the above RNA transport elements also may be incorporated into the structural protein expression cassettes, packaging cell lines, or producer cell lines described in Examples 6 and 7.

15 In yet another modification of prototype ELVS vectors, expression of the alphavirus replicons can be driven from an RNA polymerase I promoter. Briefly, because RNA polymerase I promoters are not tissue specific and are expressed in essentially all human cells in the body, they provide an attractive alternative for plasmid DNA-directed alphavirus replicon expression in transfected cells. For example, the  
20 human rDNA promoter (plasmid prHU3, Learned and Tjian, *J. Mol. Appl. Gen.*, 1:575-584, 1982), has been used to construct a vector for heterologous gene expression (Palmer et al., *Nuc. Acids Res.* 21:3451-3457, 1993).

Thus, within one embodiment of the invention a RNA polymerase I promoter can be juxtaposed with the 5' end of the replicon cDNA such that the first  
25 nucleotide transcribed in transfected cells corresponds to the authentic alphavirus 5' end. Identification of the RNA polymerase I promoter (e.g., plasmid prHU3) nucleotide at which transcription initiation occurs is determined as described previously (Dubensky et al., W/O 95/07994).

All modifications described herein can be performed with ELVS  
30 constructs containing the Sindbis virus wild-type or SIN-1 nsPs, or nsP genes from any

alphavirus. For example, all of the constructions provided in Example 5 can also be performed with plasmid pBG/SIN-1 ELVS 1.5- $\beta$ gal, whose construction is described in Example 4.

5

### EXAMPLE 6

#### CONSTRUCTION OF ALPHAVIRUS PACKAGING CELL LINES

In the present invention, alphavirus packaging cell lines (PCL) are provided, whereby the virus-derived structural proteins necessary for RNA packaging and formation of recombinant alphavirus vector particles are encoded by one or more stably transformed structural protein expression cassette(s). Synthesis of these proteins preferably occurs in an inducible manner, and in particularly preferred embodiments, via transcription of subgenomic mRNA from their native "junction region" promoter. Inducible subgenomic transcription is mediated by the input alphavirus vector RNA itself (Figure 13). Following primary transcription from the structural protein expression cassette(s), cytoplasmic amplification of the RNA transcript is initiated by vector-encoded nonstructural proteins, and ultimately leads to transcription from the junction region promoter and high level structural protein expression. The structural protein expression cassettes may include any of the previously described elements of the present invention, including RNA transport elements (*e.g.*, HBV PRE and MPMV CTE) and splicing sequences. Such PCL and their stably transformed structural protein expression cassettes can be derived using methods described within PCT application WO 95/07994, or using novel approaches described within this invention. PCL may be derived from almost any existing parental cell type, including both mammalian and non-mammalian cells. Preferred embodiments for the derivation of PCL are cell lines of human origin.

#### A. Construction of Vector-Inducible Alphavirus PCL

For example, an alphavirus structural protein expression cassette was constructed, whereby primary transcription from a CMV immediate early promoter produces an RNA molecule capable of efficient cytoplasmic amplification and

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structural protein expression only after translation of nonstructural replicase proteins from the vector RNA. Specifically, plasmid pDCMV-dl $\alpha$ PSIN (Dubensky *et al.*, *J. Virol.* 70:508-519, 1996), a DNA-based Sindbis defective helper (DH) vector, was modified to contain both a hepatitis delta virus (HDV) antigenomic ribozyme sequence

5 (Perotta and Been, *Nature* 350:434-436, 1991) for 3'-end RNA processing, and an SV40 small t antigen intron inserted within the region of nonstructural protein gene deletion. Due to restriction site duplications associated with insertion of the HDV ribozyme sequence, an additional plasmid from Dubensky *et al.*, (*ibid*), pDLTRSINGHDV, was used as starting material to reconstruct the modified CMV-based DH construct.

10 Plasmid pDLTRSINGHDV, an LTR-based Sindbis genomic clone containing the HDV ribozyme, was digested with *Bgl* II to remove the existing LTR promoter and Sindbis nucleotides 1-2289 (numbering according to Strauss *et al.*, *Virology* 133:92-110, 1984), treated with calf intestinal alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II™ (Bio101, San Diego, CA). The corresponding 5'-end

15 fragment with a CMV promoter was obtained by *Bgl* II digestion of the Sindbis genomic clone pDCMVSING (Dubensky *et al.*, *ibid*) and purification from a 1% agarose gel using GENECLAN II, and then ligated into the *Bgl* II-deleted pDLTRSINGHDV vector to generate the construct pDCMVSINGHDV. This CMV-based genomic plasmid with an HDV ribozyme was shown to produce infectious Sindbis virus and cytopathic

20 effect within 24 hr after transfection into BHK cells. Defective helper plasmid pDCMVdl $\alpha$ PSINGHDV, containing the HDV ribozyme, was then constructed by *Bsp*E I digestion and relegation under dilute conditions, to remove nonstructural gene sequences between nucleotides 422 and 7054. Subsequently, the SV40 intron was synthesized by PCR and inserted into the region of nonstructural protein gene deletion.

25 Amplification of the SV40 intron sequence was accomplished by standard three-cycle PCR with a 30 second extension time, using plasmid pBR322/SV40 (strain 776, ATCC #45019) as template and the following oligonucleotide primers that were designed to contain flanking *Bsp*E I or *Bam* HI sites.



Forward primer: BspSVSDF (5'-rest. site/SV40 intron seq.) (SEQ. ID. NO. 50)  
5'-TATATATCCGGA/AAGCTCTAAGGTAAATATAAAATTTTT-3'

Reverse primer: BamSVSAR (5'-rest. site/SV40 intron seq.) (SEQ. ID. NO. 51)  
5 5'-TATATAGGATCC/TAGGTTAGGTTGGAATCTAAAATACACAAAC-3'

Following amplification, the DNA fragment was purified using a QIAquick-spin PCR purification kit (Qiagen, Chatsworth, CA), digested with *BspE* I and *Bam* HI, purified from a 1.2% agarose gel using Mermaid™ (Bio101, San Diego, CA), and ligated into  
10 the defective helper plasmid pDCMV-dInsPSIN, which was also digested with *BspE* I and *Bam* HI, treated with calf intestinal alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II, to generate the construct pDCMV-intSINrbz (Figure 14). Plasmid pDCMV-intSINrbz, which also contains an SV40 promoter-driven neomycin resistance selectable marker on another portion of the plasmid, was  
15 transfected into BHK cells using Lipofectamine™ (Gibco/BRL, Gaithersburg, MD), as described by the manufacturer. Approximately 24 hr post-transfection, the cells were trypsinized and re-plated in media containing 600 ug/ml G418 (neomycin). The media was exchanged periodically with fresh G418-containing media and foci of resistant cells were allowed to grow. Cells were trypsinized and cloned by limiting dilution in 96 well  
20 tissue culture dishes, and individual cell clones were grown and expanded for screening.

Positive packaging activity for the individual clones was identified by Lipofectin™ (Gibco/BRL, Gaithersburg, MD)-transfection with Sindbis vector RNA that expresses a luciferase reporter gene (described in Dubensky et al., *ibid*), harvesting the culture supernatants at approximately 24 hr post-transfection, and assaying for the  
25 presence of packaged Sindbis-luciferase vector particles. In addition, initial transfection levels were determined by harvesting the transfected cell lysates using reporter lysis buffer (Promega, Madison, WI), and testing for the presence of luciferase activity by using luciferin substrate (Promega), as described by the manufacturer. To assay for packaged vector particles in the culture supernatants, 1 ml of undiluted, clarified  
30 supernatant was used to infect fresh BHK cell monolayers for approximately 18 hr. The cells were subsequently lysed as above, and luciferase activity was determined. The

presence of luciferase activity in the infected BHK cells was confirmation of packaged vector particles in the transfected cell supernatants. Several positive cell clones harboring integrated copies of the pDCMV-intSINrbz structural protein expression cassette, and functioning as PCL, were identified. Packaging data for two of the individual PCL clones (#10s-19 and #10s-22), that were representative of the group, are shown in Figure 15. In addition, the titer of packaged vector particles being produced was determined by transfecting an individual PCL clone (#10s-22) with SIN- $\beta$ -gal vector RNA (described in Dubensky et al., *ibid*). The culture supernatant was recovered at 48 hr post-transfection, clarified by passage through a 0.45 mm filter, and fresh BHK monolayers were infected with 10-fold dilutions of the supernatant. Approximately 14 hr post-infection, the cells were washed with PBS, fixed with 2% formaldehyde, washed again with PBS, and stained with X-gal. Vector particle units were then determined by counting individual blue-stained cells. Packaged  $\beta$ -gal vector titers from this PCL clone were approximately  $10^6$  infectious units/ml of supernatant. Vector-controlled inducibility of Sindbis structural protein expression was demonstrated by western blot analysis using a polyclonal rabbit antiserum specific for the structural proteins. Positive 10s-22 PCL and negative control BHK cell lysates were made in Lameli sample buffer, either before (U; uninduced) or after (I; induced) transfection with SIN- $\beta$ -gal vector RNA. As shown in Figure 16, the only cell lysate that showed expression of Sindbis structural proteins was the 10s-22 PCL clone after transfection with vector RNA. Differences in the apparent levels of expression between the capsid protein and envelope glycoproteins do not reflect the actual amounts of protein being made, rather, the lower stability of the envelope glycoproteins during the cell lysis procedure used for this particular experiment.

Packaging activity of the CMV-based DH construct was also highly efficient in non-mammalian cells, for example, C6/36 mosquito cells. The use of such a non-mammalian parental cell type for derivation of PCL may be particularly advantageous when the PCL are intended for subsequent use as starting material for the generation of vector producer cell lines. The advantage of this cell type is the natural ability of alphaviruses to establish a persistent infection, without the mammalian cell-

associated phenotype of inhibition of host macromolecular synthesis and resulting cytopathic effect (CPE). Thus a DNA-based alphavirus vector (Examples 4 and 5), with an appropriate selectable marker, may be stably transformed into mosquito or other non-mammalian cell-derived PCL. Figure 17A shows that both a DNA-based luciferase  
5 reporter vector and DH helper vector expressing Sindbis structural proteins, under the control of the CMV promoter, were fully functional in C6/36 cells, as demonstrated by luciferase vector packaging.

B. Construction of PCL with operably-linked selection marker

10 In other embodiments of the present invention, a selectable marker is operably linked to transcription of the alphavirus structural protein expression cassette. In preferred embodiments, this operable linkage is accomplished either by insertion of the marker into the region of nonstructural protein gene deletion, as a fusion with remaining nsP1 amino acids, or by insertion downstream of the structural protein genes,  
15 under the translational control of an internal ribosomal entry site (IRES) sequence. Again, amplification of the primary structural protein gene mRNA transcript and induction of structural protein expression is controlled by the input vector RNA molecule and its synthesized nonstructural proteins.

Specifically, for construction of the structural protein expression  
20 cassette, plasmid pBGS131 (Spratt et al., *Gene* 41:337-342, 1986; ATCC #37443) was modified to remove extraneous sequences, and to render an existing *Xho* I site within the kanamycin resistance gene non-functional. Plasmid pBGS131 was digested with *Xho* I and a synthetic double-stranded oligonucleotide linker with *Xho* I-compatible  
ends was ligated into the site. The synthetic 12-mer oligonucleotide, shown below, was  
25 designed as a partial palindrome that would anneal to itself generating *Xho* I sticky ends for ligation, and maintaining the kanamycin resistance gene open reading frame by inserting four in-frame amino acids.

dLXholinker (SEQ. ID. NO. 52)

30 5'-TCGATCCTAGGA

Insertion of this oligonucleotide resulted in a *Xho* I site-deleted plasmid, designated pBGS131dL*Xho*I. The plasmid was next digested with *Bsp*H I and religated to itself under dilute conditions to remove 829 bp of extraneous sequence between the ColE1 replicon and kanamycin resistance marker, generating the plasmid pBGS131dlB. The *Bsp*H I site next was changed to a *Pac* I site by digesting pBGS131dlB with *Bsp*H I, making the termini blunt with Klenow enzyme and dNTPs, and ligating with excess *Pac* I linker.

10 *Pac* I linker (SEQ. ID. NO. 53)

5'-GCTCTTAATTAAGAGC

This new construct, designated pBGS131dlB-P, was further modified by digesting with *Fsp* I and *Pvu* II to remove an additional 472 bp, including the multiple cloning site (MCS) and purifying the remaining vector from a 1% agarose gel using GENECLAN II. A replacement MCS was inserted into the modified vector by annealing two complimentary oligonucleotides, PME.MCSI and PME.MCSII, and ligating with the linear plasmid.

20 PME.MCSI (SEQ. ID. NO. 54)

5'-CTGTTTAAACAGATCTTATCTCGAGTATGCGGCCGCTATGAATTCGTTTAAACGA-3'

PME.MCSII (SEQ. ID. NO. 55)

5'-TCGTTTAAACGAATTCATAGCGGCCGCATACTCGAGATAAGATCTGTTTAAACAG-3'

25

The new, approximately 2475 bp, cloning vector was designated pBGSVG, and contained the following multiple cloning site: <*Pme* I - *Bgl* II - *Xho* I - *Not* I - *Eco*R I - *Pme* I>. Insertion of the structural protein expression cassette containing an operably linked selectable marker proceeded stepwise, as follows. A DNA fragment comprising the 3'-end of Sindbis virus, a synthetic A<sub>40</sub> tract, the antigenomic HDV ribozyme, and a BGH transcription termination signal, was removed

30

from plasmid pBG/SIN-1 ELVS 1.5 (Example 5) by digestion with *Not* I and *EcoR* I, and purification from a 1% agarose gel using GENECLAN II. Plasmid pBGSVG also was digested with *Not* I and *EcoR* I, purified from a 1% agarose gel using GENECLAN II, and ligated with the purified 3'-end/A40/HDV/BGH fragment, to  
5 generate the construct pBGSV3'. Next, an approximately 9250 bp Sindbis cDNA fragment, containing the structural protein genes and much of the nonstructural protein-encoding region, was removed from plasmid pDLTRSING (Dubensky *et al.*, *ibid*) by digestion with *Bgl* II and *Fsp* I, and purified from a 0.7% agarose gel using GENECLAN II. The Sindbis cDNA fragment was then ligated into plasmid  
10 pBGSV3', which was also digested with *Bgl* II and *Fsp* I, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The new construct was designated pBGSV3'BF. Subsequently, this construct was digested with *Bgl* II, treated with alkaline phosphatase, and purified with GENECLAN II for insertion of remaining 5'-end and nonstructural gene sequences, along with a CMV IE  
15 promoter. The remaining sequences were obtained by digestion of plasmid pDCMVSING (Dubensky *et al.*, *ibid*) with *Bgl* II, purification of the fragment from a 1% agarose gel using GENECLAN II, and ligation with the linear pBGSV3'BF vector, to create the CMV-driven Sindbis genomic construct, pBGSVCMVgen. Functionality of this construct for initiation of the Sindbis virus replication cycle was  
20 determined by Lipofectamine-mediated transfection of pBGSVCMVgen plasmid into BHK cells, and the observance of CPE within 24 hr post-transfection.

Plasmid pBGSVCMVgen was subsequently used to construct a DH structural protein expression cassette by deleting most of the nonstructural protein gene sequences and inserting a neomycin resistance gene as an in-frame fusion with  
25 remaining codons of the nsP1 open reading frame. Briefly, the neomycin resistance gene was amplified by standard three-cycle PCR from the pcDNA3 vector (Invitrogen, San Diego, CA), using the following oligonucleotide primers that were designed to contain flanking *BspE* I and *BamH* I sites.

Forward primer: NEO5'FUSE (5'-rest. site/neo gene) (SEQ. ID. NO. 56)

5'-ATATATCCGGA/GTCCGGCCGCTTGGGTGGAGAGGCTA

Reverse primer: NEO3'BAM (5'-rest. site/neo gene) (SEQ. ID. NO. 57)

5 5'-ATATAGGATCC/TCAGAAGAACTCGTCAAGAAGGCGA

Following amplification, the DNA fragment was purified with QIAquick-spin, digested with *BspE* I and *BamH* I, purified using GENECLAN II, and ligated into plasmid pBGSVCMVgen that had also been digested with *BspE* I and *BamH* I, treated with  
10 alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting construct was designated pBGSVCMVdlneo, and is shown schematically in Figure 14. The configuration of pBGSVCMVdlneo includes, as part of the structural protein expression cassette and controlled by the same CMV promoter, a fusion protein comprising the initiator methionine and amino-terminal 121 amino acids of nsP1 and  
15 the neomycin resistance gene lacking its methionine initiator codon and next ten amino acids.

Plasmid pBGSVCMVdlneo was transfected into BHK cells using Lipofectamine, as described by the manufacturer. Approximately 24 hr post-transfection, the cells were trypsinized and re-plated in media containing 600 µg/ml of  
20 the drug G418 (neomycin). The media was exchanged periodically with fresh G418-containing media and foci of resistant cells were allowed to grow. Cells were trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones were grown and expanded for screening. Positive packaging activity for the individual clones was identified by transfecting with Sindbis luciferase  
25 vector RNA and assaying for the presence of packaged Sindbis-luciferase vector particles as described in the previous section. Several positive cell clones harboring integrated copies of the pBGSVCMVdlneo structural protein gene expression cassette, and functioning as PCL, were identified. SNBST<sup>TM</sup>-luciferase vector packaging data for individual clones (F11, F13, F15) that are representative of the group, as well as the  
30 previously described 10s-22 PCL line, are shown in Figure 18.

In addition to demonstrating functional packaging activity with Sindbis-luciferase vectors, additional experiments performed using the same PCL also showed that vectors derived from other alphaviruses also could be packaged. For example, both Sindbis (Dubensky et al., *ibid.*) and Semliki Forest (pSFV3-*lacZ*; GIBCO BRL, Gaithersburg, MD) vector RNAs expressing  $\beta$ -galactosidase, were transfected into the F15 packaging cell line. Approximately 48 hr post-transfection, the culture supernatants were harvested, clarified, diluted serially, and used to infect fresh BHK cell monolayers for determination of vector particle titers. At 18 hr post-infection, the BHK cells were fixed, stained with X-gal, and the blue-staining cells were counted, as described previously. The vector titers obtained for Sindbis  $\beta$ -gal were approximately  $5 \times 10^6$  IU/ml, while the titers for SFV  $\beta$ -gal were approximately  $4 \times 10^6$  IU/ml. These data demonstrate that the two different alphaviruses and their corresponding vectors have similar packaging signals and that PCL derived for the Sindbis systems described herein are fully functional when used with another alphavirus.

Packaging activity of the pBGSVCMVdneo construct also was highly efficient in cells of human origin, for example, 293 cells. The use of such a human parental cell type for derivation of PCL may be particularly advantageous in the generation of complement resistant recombinant alphavirus particles. Figure 17B shows that both RNA and DNA-based luciferase reporter vectors were efficiently packaged following transfection into G418 resistant, pBGSVCMVdneo-transformed 293 PCL, as demonstrated by supernatant transfer of luciferase expression into BHK cells.

Another selectable drug-resistance marker also was shown to function in a similar PCL configuration, as a fusion protein with remaining nsP1 amino acids at its N-terminus. Briefly, the hygromycin phosphotransferase gene (hygromycin resistance marker, *hygro*<sup>r</sup>) was substituted into plasmid pBGSVCMVdneo, in place of the existing neomycin resistance marker. The *hygro*<sup>r</sup> gene was amplified by standard three-cycle PCR from plasmid p3'SS (Stratagene, La Jolla, CA), using the following oligonucleotide primers that were designed to contain flanking *EcoRV* and *BamHI* sites.

Forward primer: 5'HYGROEV (5'-rest. site/hygro gene) (SEQ. ID. NO. 112)

5'-TATATGATATC/AAAAAGCCTGAACTCACCGCGACG

Reverse primer: 3'HYGROBA (5'-rest. site/hygro gene) (SEQ. ID. NO. 113)

5'-ATATAGGATCC/TCAGTTAGCCTCCCCCATCTCCCG

Following amplification, the DNA fragment was purified with QIAquick-spin, digested with *EcoRV* and *BamHI*, purified using GENECLAN, and ligated into plasmid pBGSVCMVdneo that had been digested with *BspEI*, blunt-ended with Klenow, digested further with *BamHI*, treated with alkaline phosphatase, and purified from a 0.7% gel using GeneClean. The resulting construct was designated pBGSVCMVdhyg.

Plasmid pBGSVCMVdhyg was transfected into BHK cells using Lipofectamine, as described by the manufacturer. Approximately 24 hr post-transfection, the cells were trypsinized and re-plated in media containing 1.2 mg/ml of the drug hygromycin (Boehringer Mannheim). The media was exchanged periodically with fresh hygromycin-containing media and foci of resistant cells were allowed to grow into a pool. Functionality of the selected packaging cells was demonstrated by transfecting with Sindbis luciferase vector RNA and assaying for the presence of packaged Sindbis-luciferase vector particles as described in the previous section. Positive results from these packaging experiments are shown in Figure 39.

In an alternative packaging cell line structural protein expression cassette, the selectable marker (in this case neomycin resistance) was inserted downstream of the Sindbis structural protein genes and under the translational control of an internal ribosome entry site (IRES). Thus, transcription of the mRNA encoding neomycin resistance occurs both at the genomic level (from the RSV promoter) and also from the subgenomic junction region promoter. Additional features unique to this construct include the Rous sarcoma virus (RSV) LTR promoter for primary transcription and a tRNA<sup>Asp</sup> 5'-end sequence derived from Sindbis defective-interfering RNA clone DI25 (Monroe and Schlesinger, *Proc. Natl. Acad. Sci. USA* 80:3279-3283, 1983). This particular PCL expression cassette configuration was designated



987DHBBNeo, and is shown schematically in Figure 14. Specifically, plasmid 987DHBBNeo is constructed stepwise using the modified plasmid vector pBGS131dlB-P (described above) as starting material. A cDNA fragment containing the junction region promoter, the structural protein gene sequences, and 3'-untranslated region +  
 5 polyA is obtained by digestion of the full-length Sindbis cDNA clone pRSING (Dubensky et al., *ibid*) with *Bam*H I and *Xba* I, and purification from a 0.7% agarose gel using GENECLAN II. The Sindbis cDNA DNA fragment is ligated with plasmid vector pBGS131dlB-P that also has been digested with *Bam*H I and *Xba* I, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II, to  
 10 generate the construct pBGSINsp.

Next, the transcription termination signal from the SV40 early region is inserted between the *Sac* I and *Eco* RI sites of pBGSINsp, immediately downstream of the Sindbis sequence. The SV40 viral nucleotides 2643 to 2563, containing the early region transcription termination sequences, are isolated by PCR amplification using the  
 15 primer pair shown below and the pBR322/SV40 plasmid (ATCC # 45019), as template.

Forward primer: FSVTT2643 (5'-rest. site/SV40 nts 2643-2613) (SEQ. ID. NO. 58)

5'-TATATATGAGCTCTTACAAATAAAGCAATAGCATCACAAATTTC

20 Reverse primer: RSVTT2563 (rest. site/SV40 nts 2563-2588) (SEQ. ID. NO. 59)

5'-TATATGAATTCGTTTGGACAAACCACAACCTAGAAATG

The primers are used in a standard three-cycle PCR reaction with a 30 second extension period. The amplification products are purified with QIAquick-spin,  
 25 digested with *Sac* I and *Eco* RI, purified again with the Mermaid kit, and the 90 bp fragment is ligated into plasmid pBGSINsp that also has been digested with *Sac* I and *Eco* RI, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. This construction is known as pBGSINspSV.

Next the RSV promoter and Sindbis 5'-end sequences, including the DI  
 30 tRNA<sup>Asp</sup> structure, are assembled by overlapping PCR and the entire fragment is

inserted into the structural protein gene vector pBGSINspSV. In PCR reaction #1, the RSV promoter fragment is amplified by standard three cycle PCR, with a 1 minute extension, using the following oligonucleotide primers that are designed to also contain a flanking *Bgl* II site in one primer and sequences overlapping the tRNA 5'-end in the other. The Sindbis tRNA 5'-end is positioned immediately adjacent to the RSV promoter transcription start site.

Forward primer: 5'RSVpro (5'-rest. site/RSV seq.) (SEQ. ID. NO. 60)

5'-TATATAGATCT/AGTCTTATGCAATACTCTTGTAGT

10

Reverse primer: 3'RSVtR (5'-Sin tRNA seq/RSV seq.) (SEQ. ID. NO. 61)

5'-GGGATACTCACCCTATATCTCGACGGTATCGAGGTAGGGCACT

In PCR reaction #2, the Sindbis 5'-end plus tRNA sequence is amplified by standard three cycle PCR with a 1 minute extension, from template plasmid Toto1101(5'tRNA<sup>Asp</sup>) (Bredenbeek et al., *J. Virol.* 67:6439-6446, 1993), using the following oligonucleotide primers that are designed to also contain a flanking *Bam*H I site in one primer and sequences overlapping the 3'RSVtR primer in the other.

20 Forward primer: 5'tRNASin (5'-Sindbis + tRNA seq. only) (SEQ. ID. NO. 62)

5'-GATATAGTGGTGAGTATCCCCG

Reverse primer: 3'SinBam (3'-rest. site/Sindbis seq.) (SEQ. ID. NO. 63)

5'-TATATGGATCC/CGGAGATCCTTAATCTTCTCATG

25

Following amplification, the DNA fragments are purified with QIAquick-spin and used together as templates in a subsequent three-cycle PCR reaction with 2 minute extension, using additional 5'RSVpro and 3'SinBam primers. The resulting overlapping PCR amplicon is purified using GENECLAN II, digested with *Bgl* II and *Bam*H I, and ligated into plasmid pBGSINspSV that also has been digested with *Bgl* II and *Bam*H I,

30

treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting structural protein expressing, defective helper is designated 987DHBB.

Next, the IRES sequence from encephalomyocarditis virus (EMCV), is  
5 positioned immediately upstream of the neomycin phosphotransferase gene, as a  
selectable marker, by overlapping PCR, and the entire amplicon is inserted into the *Nsi*  
*I* site of 987DHBB. Insertion at the *Nsi I* site will position the selectable marker  
immediately downstream of the structural protein ORF. In PCR reaction #1, the EMCV  
IRES fragment (nucleotides 260-827) is amplified by standard three cycle PCR, with a  
10 30 second extension, from template plasmid pBS-ECAT (Jang et al., *J. Virol* 63:1651,  
1989), using the following oligonucleotide primers that are designed to also contain a  
flanking *Nsi I* site in one primer and sequences overlapping the neo gene in the other.

Forward primer: 5'EMCVIRES (5'-rest. site/EMCV seq.) (SEQ. ID. NO. 64)

15 5'-TATATATGCAT/CCCCCCCCCCCCCAACG

Reverse primer: 3'EMCVIRES (5'-pcDNA + neo seq/EMCV seq.) (SEQ. ID. NO. 65)

5'-CATGCGAAACGATCCTCATC/CTTACAATCGTGGTTTTCAAAGG

20 In PCR reaction #2, the neo resistance marker is amplified by standard three cycle PCR  
with a 1.5 minute extension, from template plasmid pcDNA3 (Invitrogen, San Diego,  
CA), using the following oligonucleotide primers that are designed to also contain a  
flanking *Nsi I* site in one primer and sequences overlapping the 3'EMCVIRES primer in  
the other.

25

Forward primer: 5'Neo/pcDNA (5'-pcDNA + neo seq. only) (SEQ. ID. NO. 66)

5'-GATGAGGATCGTTTCGCATGATTGA

Reverse primer: 3'Neo/pcDNA (3'-rest. site/neo seq.) (SEQ. ID. NO. 67)

30 5'-TATATATGCAT/TCAGAAGAACTCGTCAAGAAGGCGA

Following amplification, the DNA fragments are purified with QIAquick-spin and used together as templates in a subsequent three-cycle PCR reaction with 2 minute extension, using additional 5'EMCVIRES and 3'Neo/pcDNA primers. The resulting overlapping  
 5 PCR amplicon is purified using GENECLAN II, digested with *Nsi* I, and ligated into plasmid 987DHBB that also has been digested with *Nsi* I, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting structural protein expression construct, with the IRES/neo insert in the Sindbis 3'-untranslated region, is designated 987DHBBNeo.

10 To generate stable packaging cell lines, BHK cells were transfected with 10 ug of plasmid 987DHBBNeo, using a standard calcium phosphate precipitation protocol. Approximately 24 hr post-transfection, the media was replaced with fresh media containing 1 mg/ml of the drug G418. After one additional day, the cells were trypsinized and re-plated at 1/10 density in media containing 500 ug/ml G418. After  
 15 several more passages, the cells were subjected to dilution cloning and individual clones were expanded. The ability of individual clones to function as packaging cell lines was determined by calcium phosphate transfection of plasmid RSV/Sinrep/LacZ, a Sindbis DNA vector expressing  $\beta$ -gal, and assaying for the presence of packaged vector particles in the supernatants after 48 hr. The packaged vector replicons were titered by  
 20 the CPE assay described in Frolov and Schlesinger (*J. Virol.* 68:1721-1727, 1994) and one that gave high titers of packaged particles, designated 987DH-BBNeo, was used for further characterization. Packaged vector titers were determined at 48 hr, following transfection of either RNA- or DNA-based Sindbis vectors expressing  $\beta$ -gal, using several different transfection techniques. The results were as follows:

25

<u>transfection procedure</u>	<u>nucleic acid added</u>	<u>titers (infectious units/ml)</u>
electroporation	RSVSINrep/LacZ DNA (2.5 ug)	$1.5 \times 10^9$ /ml
electroporation	SINrep/LacZ RNA (2.5 ug)	$6 \times 10^9$ /ml
Lipofectamine	RSVSINrep/LacZ DNA (2 ug)	no packaged particles
Lipofectin	SINrep/LacZ RNA (2 ug)	$5-6 \times 10^7$ /ml

<u>transfection procedure</u>	<u>nucleic acid added</u>	<u>titers (infectious units/ml)</u>
Calcium Phosphate	RSVSINrep/LacZ DNA (10 ug)	$1.5 \times 10^9/\text{ml}$

In addition, SINrep/LacZ particles that were packaged using 987DH-BB cell lines subsequently were used to infect fresh BHK cell monolayers and examine both RNA and protein expression patterns. Figure 19 shows the RNA pattern after

5 BHK cells were infected with two different preparations of SINrep/LacZ particles at a MOI of 150 infectious units per cell (lanes 1 and 2), or wild-type Sindbis virus (lane 3), as a control. Seven hours post-infection, dactinomycin (1 ug/ml) and [<sup>3</sup>H]uridine (20 uCi/ml) were added, followed by harvest and analysis of RNA 4 hr later, according to Bredenbeek et al. (*J. Virol.* 67:6439-6446, 1993). The high MOI was used in order to

10 detect possible recombinants. Horizontal lines to the right of the gel lanes indicate the Sindbis and  $\beta$ -gal RNAs of interest. The highest molecular weight band indicates the genomic RNA of the replicon or virus (lanes 1 and 2, SINrep/LacZ; lane 3 Sindbis virus). The next two RNAs indicated are the genomic RNA of the 987DH-BBNeo PCL expression cassette and the inducible subgenomic structural protein mRNA from the

15 same 987DH-BBNeo PCL cassette. The presence of the latter two bands demonstrates that the helper genomic RNA derived from the packaging cell line is also co-packaged. The next RNA bands, those present in greatest abundance, are the subgenomic RNAs derived from either SINrep/LacZ (lanes 1 and 2) or the Sindbis virus genome (lane 3).

Protein analysis was performed following infection of BHK 21 cells with

20 packaged SINrep/LacZ replicons at a MOI of 20 infectious units/cell. Fifteen hours post-infection, the cells were labeled with [<sup>35</sup>S] methionine for 30 minutes, lysates made, and the proteins analyzed by SDS-PAGE. As shown in Figure 20 (lanes 2 and 3), both beta-galactosidase and the Sindbis virus capsid protein are labeled in the vector particle-infected cells, but not in uninfected cells (lane 1). The presence of capsid

25 shows that some of the packaged particles also contain structural protein gene RNA transcripts from the PCL.

C. Construction of "split structural gene" PCL configurations

In other embodiments of the present invention, PCL are provided wherein the alphavirus structural proteins are expressed, not as a polyprotein from a single mRNA, with its native post-translational processing, but rather, as separate  
5 proteins from independent mRNAs that are transcribed via multiple cassettes. Such a configuration greatly minimizes the possibility of recombination or co-packaging events that lead to formation of replication-competent or infectious virus. In preferred embodiments, the capsid protein is expressed from one stably transformed cassette and the envelope glycoproteins are expressed together from a second stably transformed  
10 cassette, and each is expressed in a vector-inducible manner from the junction region promoter (described above).

For example, the Sindbis virus capsid protein gene was amplified from plasmid pDLTRSING (Dubensky et al., *ibid*), by standard three-cycle PCR with a 1.5 minute extension, using the following oligonucleotide primers that were designed to  
15 contain a flanking *Xho* I site and capsid protein gene initiation codon or a flanking *Not* I site and translation stop codon.

Forward primer: SIN5'CXho (5'-rest. site/capsid seq.) (SEQ. ID. NO. 68)

5'-ATATACTCGAG/ACCACCACCATGAATAGAGGATTC

20

Reverse primer: SIN3'CNot (5'-rest. site/stop codon/capsid seq.) (SEQ. ID. NO. 69)

5'-TATATGCGGCCGC/TATTA/CCACTCTTCTGTCCCTTCCGGGGT

Following amplification, the capsid DNA fragment was purified with QIAquick-spin,  
25 digested with *Xho* I and *Not* I, purified using GENECLAN II, and ligated into the DNA-based Sindbis expression vector pDCMVSIN-luc (Dubensky et al., *ibid*), that also had been digested with *Xho* I and *Not* I to remove its luciferase reporter gene insert, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting capsid protein expression construct was designated  
30 pDCMVSIN-C. Plasmid pDCMVSIN-C was subsequently digested with *BspE* I to

remove most nonstructural protein gene sequences, and re-ligated to itself under dilute conditions to create the DH vector construct, pDCMVSINdl-C.

Plasmid pDCMVSINdl-C, which also contains a neomycin resistance cassette, was transfected into BHK cells using Lipofectamine, as described by the  
5 manufacturer. Approximately 24 hr post-transfection, the cells were trypsinized and re-plated in media containing 600 ug/ml of the drug G418 (neomycin). The media was exchanged periodically with fresh G418-containing media and foci of resistant cells were allowed to grow. Cells were trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones were grown and expanded for screening.  
10 Cells which inducibly expressed capsid protein in response to input vector were identified by transfecting with Sindbis luciferase vector RNA, making cell lysates approximately 15 hr post-transfection, and performing western blot analysis with a rabbit anti-Sindbis polyclonal antibody. Several positive cell clones harboring integrated copies of the capsid protein gene expression cassette and inducibly  
15 expressing the protein were identified.

In order to demonstrate both inducibility and functionality of the expressed capsid in the context of "split structural gene" cassettes, an additional construct that expressed the Sindbis virus envelope glycoproteins was generated from pDCMVSIN-luc. Briefly, the Sindbis envelope glycoprotein genes were amplified from  
20 plasmid pDLTRSING by standard three-cycle PCR, with a 2.5 minute extension, and using the following oligonucleotide primers that are designed to contain a flanking *Xho* I site and translation initiation codon in good Kozak context, or a flanking *Not* I site and the translation stop codon.

25 Forward primer: 5'GLYCO-X (5'-rest. site/initiation codon/glycoprotein seq.)  
(SEQ. ID. NO. 70)

5'-ATATACTCGAG/AGCAATG/TCCGCAGCACCACTGGTCACGGCA

Reverse primer: 3'GLYCO-N (5'-rest. site/glycoprotein seq.) (SEQ. ID. NO. 71)

30 5'-ATATAGGCGGCCGC/TCATCTTCGTGTGCTAGTCAGCATC

Following amplification, the glycoprotein gene DNA fragment was purified with QIAquick-spin, digested with *Xho* I and *Not* I, purified using GENECLAN II, and ligated into the DNA-based Sindbis expression vector pDCMVSIN-luc, that also had  
5 been digested with *Xho* I and *Not* I to remove its luciferase reporter gene insert, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting glycoprotein expression construct was designated pDCMVSIN1.5PE.

Figure 21 is a western blot demonstrating vector controlled inducibility of two different clonal capsid lines (9-3 and 9-9) cell lines, that were transfected with  
10 Sindbis DNA vectors expressing the envelope glycoproteins (1.5PE lanes) or a  $\beta$ -galactosidase reporter, pDCMVSIN- $\beta$ -gal (Dubensky et al., *ibid* ; 1.5 $\beta$ -gal lanes), or "mock" transfected (M lanes), using Lipofectamine. Cell lysates were made at 48 hr post-transfection, separated by SDS-PAGE, and transferred to membranes, where they were probed with a combination of antibodies specific for Sindbis structural proteins  
15 and  $\beta$ -galactosidase. The blot clearly shows the inducibility of capsid protein in response to the nonstructural proteins supplied by either vector, as well as the expression of  $\beta$ -galactosidase and the envelope glycoproteins. Functionality of the "split structural gene" capsid cell lines, by complementation and vector particle packaging, was demonstrated by co-transfecting the  $\beta$ -galactosidase and envelope  
20 glycoprotein vectors into a capsid cell line using Lipofectamine, and assaying for packaged particles in the culture supernatants. Approximately 48 hr post-transfection, the supernatants were harvested and clarified for the packaging assays and vector titer determination. In addition, the cells were lysed using Lameli sample buffer and examined by western blot analysis with polyclonal anti-Sindbis antibody, demonstrating  
25 expression of both capsid protein and the vector supplied envelope glycoproteins. The supernatants were then tested for the presence of packaged vector particles by infecting naive BHK cells for approximately 18 hr, and staining for  $\beta$ -gal reporter gene expression, as described previously in this example. Functionality of the cell lines for complementation and packaging was demonstrated by the observance of blue-stained  
30  $\beta$ -gal expressing cells.



To generate stable "split structural gene" PCL that have separate vector inducible expression cassettes for both capsid protein and the envelope glycoproteins, the above described capsid cell lines are used, in conjunction with an additional envelope glycoprotein expression construct that contains a different selectable marker (for example, hygromycin B resistance). Briefly, plasmid pBG/SIN-1 ELVS 1.5- $\beta$ -gal (Example 5) is digested with *Bam*H I and *Fsp* I to isolate the sequences comprising the junction region promoter, the  $\beta$ -gal reporter gene, and some 3'-end sequences. The desired fragment is purified from a 1% agarose gel using GENECLAN II, and ligated into plasmid pBGSVCMVdlneo (see above) that also has been digested with *Bam* HI and *Fsp* I to eliminate all structural protein gene sequences, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting construct is designated as pBGSVCMVdlsP-luc. Plasmid pBGSVCMVdlsP-luc is next digested with *Xho* I and *Not* I to remove the luciferase reporter gene, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II, and the *Xho* I- and *Not* I-digested envelope glycoprotein PCR amplicon from above is subsequently ligated into the digested pBGSVCMVdlsP-luc vector to generate the envelope glycoprotein expressing DH construct, pBGSVCMVdl-G. Insertion of a hygromycin resistance marker cassette into this plasmid, as well as flanking HSV TK promoter and polyadenylation sequences, is accomplished by PCR amplification, using a standard three-cycle protocol with 2.5 minute extension, plasmid pDR2 (Clontech, Palo Alto, CA) as template, and the following oligonucleotide primers that are designed to contain flanking *Pac* I sites.

Forward primer: 5'HYGRO/Pro-P (5'-rest. site/pDR2 seq.) (SEQ. ID. NO. 72)

5'-ACACATTAATTAA/CGATGCCCGCCGGAAGCGAGAA

Reverse primer: 3'HYGRO/pA-P (5'-rest. site/pDR2 seq.) (SEQ. ID. NO. 73)

5'-ACACATTAATTAA/GTATTGGCCCCAATGGGGTCT

Following amplification, the DNA fragment is purified with QIAquick-spin, digested with *Pac* I, purified using GENECLAN II, and ligated into plasmid pBGSVCMVdl-G that also has been digested with *Pac* I, treated with alkaline phosphatase, and purified using GENECLAN II. The resulting construct is designated as pBGSVhygro-G.

5 Plasmid pBGSVhygro-G, which now also contains a hygromycin resistance cassette, is transfected into the capsid cell line using Lipofectamine, as described by the manufacturer. Approximately 24 hr post-transfection, the cells are trypsinized and re-plated in media containing 1200 ug/ml of hygromycin (Calbiochem, La Jolla, CA). The media is exchanged periodically with fresh hygromycin-containing  
10 media and foci of resistant cells are allowed to grow. Cells are trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones are grown and expanded for screening. Cells which inducibly express biologically active capsid protein and envelope glycoproteins in response to input vector are identified in two ways: first, by Lipofectin-mediated transfection with Sindbis luciferase vector  
15 RNA and probing 15 hr post-transfection lysates in western blots, as described above; and second, by similar Sindbis luciferase vector transfection, but harvesting the supernatants at 24 hr post-transfection, and testing for the transfer of luciferase expression to fresh BHK cells by infection with the supernatants (see above). Split structural gene PCL derived in this manner are designated C/GLYCO PCL.

20

D. Construction of PCL with "hybrid" structural proteins

An additional approach which may be utilized to decrease the level of co-packaging or recombination between DH and vector RNA molecules, to enhance  
translation of the glycoprotein genes, or to alter the cell or tissue specificity of the  
25 packaged recombinant alphavirus vector particles, makes use of structural protein genes derived from other alphaviruses or togaviruses. More specifically, numerous combinations of alphavirus or togavirus structural protein genes for use with Sindbis virus or different alphavirus vectors can be envisioned. For example, the capsid protein gene of Ross River virus (RRV), may be used in conjunction with the envelope  
30 glycoprotein genes of Sindbis virus (expressed from the same or a different construct), to package a Sindbis virus-derived vector described in examples 3, 4, or 5. In addition,

a deleted form of the RRV capsid protein gene may be positioned immediately upstream of the Sindbis glycoprotein gene sequences to serve as a translational enhancer elements. As another example, the structural proteins of Sindbis virus may be used to package Semliki Forest virus RNA vectors.

5 Specifically, defective helper (DH) structural protein constructs that contain an intact or deleted form of the RRV capsid gene (Figure 22), plus the Sindbis glycoprotein genes, were constructed by PCR amplification of Sindbis virus or Ross River virus sequences from plasmid templates Toto1101 (Rice et al., *J. Virol.* 61:3809-3819, 1987) and RR6415 (Kuhn et al., *Virology* 182:430-441, 1991), respectively, and  
 10 incorporation of those sequences into the previously described DH constructs DH-BB or DH-BB(5'SIN) (Bredenbeek et al., *J. Virol.* 67:6439-6446, 1993), for transcription *in vitro* with SP6 polymerase. The DH constructs DH-BB or DH-BB(5'SIN) different by the presence (DH-BB) or absence (DH-BB(5'SIN)) of a DI-derived tRNA<sup>Asp</sup> sequence at the Sindbis 5'-end. The following table indicates the specific PCR primer  
 15 sequences and their corresponding Sindbis or Ross River nucleotide positions, with capital letters indicating viral nucleotides and lower case letters indicating additional nucleotides of restriction sites used in cloning steps or specific point mutations. Point mutations in primer 6 (RRV Bsp) did not change the resulting amino acid sequence.

20 Primers used for PCR:

1. RRV Ava: (nt.7510-7525) (SEQ. ID. NO. 74)

5'-ccacgaattcGGTCCTAAATAGATGC

25 2. RRV Nci1: (nt.7606-7620) (SEQ. ID. NO. 75)

5'-ccacaagcttCCGGGCGAGGCCGCC

3. RRV Nci2: (nt.7616-7630) (SEQ. ID. NO. 76)

5'-ccacggatCCCGGCGTTCCGTCC

4. RRV Apa1: (nt.8088-8102) (SEQ. ID. NO. 77)  
5'-ccacaagcttGTGCACTGGGATCTG
5. RRV Apa2: (nt.8097-8111) (SEQ. ID. NO. 78)  
5'-ccacggatccGTGCACATGAAGTCC
6. RRV Bsp: (nt.8339-8361) (SEQ. ID. NO. 79)  
5'-ccacaagCTTCcGGaGTTACCCGAGTGACC
- 10 7. RRV Afl1: (nt.7820-7836) (SEQ. ID. NO. 80)  
5'-ccaccttaaGCGTCGGCTTTTCTTC
8. RRV Afl2: (nt.7892-7907) (SEQ. ID. NO. 81)  
5'-ccaccttaaGAGAAGAGAAAGAATG
- 15 9. SIN Ava: (nt.7591-7594) (SEQ. ID. NO. 82)  
5'-ccacaagcttGGACCACCGTAGAG
10. SIN Bam: (nt.7325-7343) (SEQ. ID. NO. 83)  
20 5'-CCGCGTGGCGGATCCCCTG
11. SIN Bsp: (nt.8418-8433) (SEQ. ID. NO. 84)  
5'-ccacggatCCGGAAGGGACAGAAG
- 25 12. SIN Bsu: (nt.8887-8902) (SEQ. ID. NO. 85)  
5'-CACGGTCCTGAGGTGC

30 PCR reactions were performed using the primer pairs indicated below, in a standard three cycle protocol, with 30 sec extensions and Vent polymerase, to produce the corresponding DNA fragments, which are also indicated below.

PCR fragments: primer pairs, plasmid template

Fragment 1: pr1+pr2, RRV6415 plasmid

Fragment 2: pr3+pr4, RRV6415 plasmid

5 Fragment 3: pr5+pr6, RRV6415 plasmid

Fragment 4: pr3+pr7, RRV6415 plasmid

Fragment 5: pr4+pr8, RRV6415 plasmid

Fragment 6: pr9+pr10, Toto1101 plasmid

Fragment 7: pr11+pr12, Toto1101 plasmid

10

Following amplification, the PCR products were digested with the indicated enzymes, and ligated into the pUC18 plasmid analog, pRS2, which contains additional polylinker sites and which had also been digested with the same enzyme combinations: fragment 1 was cut with *EcoR I*+*Hind III*; fragment 2 with *BamH I*+*Hind III*; fragment 3 with  
 15 *BamH I*+*Hind III*; fragment 4 with *BamH I*+*Afl II*; fragment 5 with *Hind III*+*Afl II*; fragment 6 with *BamH I*+*Hind III*; and fragment 7 with *BamH I*+*Bsu 36I*. All insertions were sequenced to verify that artifacts had not been acquired during PCR.

Subsequently, the fragments were released from the pRS2 plasmids using the enzymes indicated below, and ligated exactly as indicated to generate the next  
 20 set of constructs. To generate FR8, fragment 6 (cut by *Bam HI* and *Ava II*) was ligated with fragment 1 (cut by *Ava II* and *Nci I*), fragment 2 (cut by *Nci I* and *Hind III*) and plasmid pRS2 (cut by *Bam HI* and *Hind III*). To generate FR9, fragment 6 (cut by *BamH I* and *Ava II*) was ligated with fragment 1 (cut by *Ava II* and *Nci I*), fragment 4  
 (cut by *Nci I* and *Afl II*) and plasmid pRS2 (cut by *BamH I* and *Afl II*). To generate  
 25 FR10, fragment 5 (cut by *Afl II* and *ApaL I*) was ligated with fragment 3 (cut by *ApaL I* and *Hind III*) and plasmid pRS2 (cut by *Afl II* and *Hind III*). After transformation of *E. coli*, plasmids were analyzed by restriction analysis and their inserts were again isolated by digestion and used for the next steps of cloning.

The FR8 insert (cut by *BamH I* and *ApaL I*) was ligated with fragment 3  
 30 (cut by *ApaL I* and *BspM II*), fragment 7 (cut by *BspM II* and *Bsu36 I*) and plasmid DH-

BB (cut by *BamH* I and *Bsu36* I). The same fragments also were used to replace the *BamH* I-*Bsu36* I fragment of plasmid DH-BB(5'SIN). The resulting plasmids were designated DH-BB Crrv and DH-BB(5'SIN) Crrv, respectively (see Figure 23). The FR9 insert (cut by *BamH* I and *Afl* II) was ligated with the FR10 insert (cut by *Afl* II and *BspM* II), fragment 7 (cut by *BspM* II and *Bsu36* I) and plasmid DH-BB (cut by *BamH* I and *Bsu36* I). The same fragments also were used to replace the *BamH* I-*Bsu36* I fragment of plasmid DH-BB(5'SIN). The resulting plasmids were designated DH-BB CΔrrv and DH-BB(5'SIN) CΔrrv (see Figure 23).

Multiple uses for DH constructs that contain chimeric structural protein genes are possible, and two such approaches are illustrated in Figures 24 and 25. In Figure 24, the intact Ross River capsid protein gene is linked with the Sindbis glycoprotein gene sequences (DH-BB Crrv or DH-BB(5'SIN) Crrv), as part of a defective helper construct, and co-transfected with a Sindbis reporter RNA vector replicon to demonstrate packaging into recombinant alphavirus particles (Figure 26). In Figure 25, the deleted form of the Ross River capsid protein gene is linked with the Sindbis glycoprotein gene sequences (DH-BB CΔrrv and DH-BB(5'SIN) CΔrrv), as a translational enhancer and part of the DH construct, while the Sindbis capsid protein gene expressed from a second DH construct. Both DH constructs are co-transfected with a Sindbis reporter RNA vector replicon to demonstrate packaging into recombinant alphavirus particles (Figure 26). Additionally, the Ross River capsid protein gene may be expressed alone from one DH construct, while the Sindbis glycoproteins are expressed from another, for use in packaging. Using this knowledge and the availability of several other alphaviruses from which to derive structural protein gene sequences, a large number of different protein combinations may be generated in similar approaches.

Alternatively, the entire complement of structural protein genes from one alphavirus, or other members of the *Togaviridae* (e.g., rubella virus) may be used to package an RNA vector derived from another, as shown above for SFV vectors and Sindbis structural proteins. In an alternative embodiment, the structural protein genes from Venezuelan equine encephalitis (VEE) virus may be used to package a Sindbis-

virus derived vector (wild-type or displaying the phenotype described in Example 4 or 5). Such a method provides recombinant alphavirus particles containing vector RNAs which exhibit the desirable properties of the present invention, such as delayed, reduced or no inhibition of host macromolecular synthesis, plus, structural proteins which redirect the tropism of the recombinant particle. Venezuelan equine encephalitis virus (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector constructs packaged by a cell line expressing the VEE structural proteins will display the same lymphotropic properties as the parental VEE virus from which the packaging cell structural protein gene cassette was obtained.

Specifically, the Trinidad donkey strain of VEE virus (ATCC #VR-69) is propagated in BHK-21 cells, and virion RNA is extracted using procedures similar to those described in Example 1. The entire structural protein coding region is amplified by PCR with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding Kozak consensus sequence, and the UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer is complementary to VEE nucleotides 11206-11186 (sequence from Kinney et al., *Virology* 170:19-30, 1989). PCR amplification of VEE cDNA corresponding to the structural protein genes is accomplished using a two-step reverse transcriptase-PCR protocol as described above, the VEE genome RNA as template, and the following oligonucleotide pair, which contain flanking *Xho* I and *Not* I sites:

Forward primer: VEE 7553F (5'-rest. site/VEE capsid seq.) (SEQ. ID. NO. 86)

5'-TATATATATCTCGAGACCGCCAAGATGTTCCCGTTCCAGCCA-3'

Reverse primer: VEE 11206R (5'-rest. site/VEE E1 glyco seq.) (SEQ. ID. NO. 87)

5'-TATATATATGCGGCCGCTCAATTATGTTTCTGGTTGGT-3'

Following PCR amplification, the approximately 3800 bp fragment is purified from a 0.7% agarose gel using GENECLAN II, and digested with *Xho* I and *Not* I. The resulting fragment is then ligated into the DNA-based Sindbis expression vector pDCMVSIN-luc (see above), that also has been digested with *Xho* I and *Not* I to remove its luciferase reporter gene insert, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting VEE structural protein expression construct is designated pDCMV-VEEsp. Plasmid pDCMV-VEEsp subsequently is digested, under limiting partial digest conditions, with *BspE* I to remove most nonstructural protein gene sequences, and re-ligated to create the structural protein-expressing DH vector construct, pDCMV-VEEdl.

Plasmid pDCMV-VEEdl, which also contains a neomycin resistance marker, is transfected into BHK cells using Lipofectamine, as described by the manufacturer. Approximately 24 hr post-transfection, the cells are trypsinized and replated in media containing 600 µg/ml of the drug G418. The media is exchanged periodically with fresh G418-containing media and foci of resistant cells are allowed to grow. Cells are trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones are grown and expanded for screening. Cells which inducibly express VEE structural proteins in response to input vector are identified by transfecting with Sindbis luciferase vector RNA, and assaying for VEE structural protein expression in cell lysates or packaged luciferase vector in the supernatants, as described previously. Structural protein genes obtained from variants of VEE, or other alphaviruses and their variants differing in tissue tropism, also are useful when following this approach. In addition, each of the various structural protein gene expression cassette configurations described in this example may be used.

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#### E. Production of packaged alphavirus vectors from PCL

Alphavirus derived PCL described throughout this example may be used in a number of different ways to produce recombinant alphavirus particle stocks, and include the introduction of vector by either transfection of RNA or DNA molecules, infection with previously produced packaged vector-containing particles, or the intracellular production of vector from stably transformed expression cassettes. The

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utility of alphavirus PCL for the production of vector particles is demonstrated first with a reporter vector construct, and later may be applied to any other vector constructs which express a desired heterologous sequence. For example, a stock of packaged Sindbis- $\beta$ -gal vector particles is obtained by electroporation of approximately  $10^7$  alphavirus C/GLYCO or other PCL cells (see above, and for example, figures 15, 17, 18) with 50 ug pKSSIN-1-BV- $\beta$ -gal or -luciferase RNA (Example 4) or pBG/SIN-1 ELVS 1.5- $\beta$ -gal or -luciferase DNA (Example 5), using the procedure described in (Liljestrom and Garoff, *Bio/Technology* 9:1356-1361, 1991). The transfected PCL are incubated at a desired temperature (e.g., 37°C), and at approximately 48 hr. post-transfection, the supernatants are harvested and clarified by passage through a 0.45 micron filter. Additional formulation may be performed using parameters illustrated in the detailed description of this invention.

Alternatively, a stock of packaged recombinant alphavirus particles (obtained using PCL as above, or by co-transfection of vector and DH constructs) is used to infect a naive culture of PCL, for further amplification. For example,  $5 \times 10^7$  of alphavirus C/GLYCO or other PCL cells are infected with a stock of packaged pKSSIN-1-BV- $\beta$ -gal vector at an approximate multiplicity of infection (MOI) of 1 infectious unit/cell. Upon reaching the cell cytoplasm, the particle delivered RNA vector is autocatalytically amplified and packaged into additional progeny particles. After incubation at the desired temperature (e.g., 37°C) for approximately 48 hr., the culture supernatants are harvested, clarified by passage through a 0.45 micron filter, and formulated as desired.

In still another method which exploits the ability of PCL to further amplify packaged recombinant alphavirus vector particles, stocks of packaged particles are used to infect naive cultures of PCL to create a working cell bank of vector-containing PCL (vector producing cells, Figure 27), which may subsequently be used to seed another naive culture of PCL. For example, such a working cell bank is obtained by infection of alphavirus C/GLYCO or other PCL cells with the packaged vector stock at a M.O.I.= 5. Approximately 2-3 hr post-infection, the vector containing PCL are gently detached and cell number is determined. The vector containing PCL may now

be used directly, or aliquoted and stored in liquid N<sub>2</sub> as a vector producing cell bank. When desired, the cells are seeded directly into a previously growing culture of naive alphavirus C/GLYCO or other PCL at a ratio of approximately 1 vector producing cell per 1000 fresh PCL, for production of large quantities of high titer packaged vector particles. Aliquots of culture supernatant are harvested at various times post-coculture to determine the time of maximal recombinant alphavirus particle production, and that time is chosen for further harvest, purification and formulation, as described above. The same sequential amplification methodology using vector producing cells also is useful for large-scale production of any desired recombinant protein (Figure 27). For the production of recombinant protein, supernatants or cell lysates may be harvested, depending on the nature of the recombinant protein.

In yet another method for producing high titer or large scale stocks of packaged recombinant alphavirus particles, the desired expression vector is introduced into 1-5% of a naive alphavirus PCL culture by transfection of *in vitro* transcribed RNA or plasmid DNA vector using a commonly accepted reagent or method (for example, Lipofectin or Lipofectamine, respectively, or infection with vector particles at low MOI [ $\leq 0.1$ ]), as described herein. The recombinant vector particles produced by the initial cells, into which vector was introduced, subsequently infect other naive packaging cells in the culture, which in turn, produce even more packaged particles. This process of temporal amplification continues until packaged recombinant alphavirus particles are produced in all cells of the PCL culture. This amplification process is demonstrated in Figure 28. ELVS 1.5- $\beta$ -gal plasmid DNA was transfected into 987DHBBNeo packaging cells or into BHK-21 cells, and the levels of  $\beta$ -galactosidase present in cell lysates was measured, as described previously, at the indicated times post-transfection. In BHK-21 cells, the level of  $\beta$ -galactosidase expression reached a maximum by approximately 48 hpt, and plateaued. In contrast, the level of  $\beta$ -galactosidase expression continued to increase over a longer period of time in the ELVS 1.5- $\beta$ -gal transfected 987DHBBNeo PCL culture, reflecting the recombinant vector particle amplification process, and the ultimate expression of  $\beta$ -galactosidase in all of the cells of the culture. In all cases, stocks of recombinant alphavirus vector particles may be

formulated so as to be pharmaceutically acceptable, using any of the methods described herein.

#### EXAMPLE 7

##### CONSTRUCTION OF ALPHAVIRUS PRODUCER CELL LINES

The generation of alphavirus PCL, as described above, coupled with the construction of DNA-based alphavirus vectors exhibiting reduced, delayed, or no inhibition of host cell macromolecular synthesis (Examples 1, 2, 4 and 5), provides a relatively straightforward mechanism to derive alphavirus vector producer cell lines. In certain embodiments of the present invention, the vector producer cell lines contain one or more stably transformed structural protein gene expression cassettes, and also alphavirus RNA expression vector molecules with the above phenotype, that are transfected, transduced, or intracellularly produced, leading to the production of packaged vector particles. In preferred embodiments, an RNA vector replicon is produced intracellularly from a stably transformed DNA molecule (eukaryotic layered vector initiation system) that exists in either an integrated form or as an episomal DNA, with transcription of vector RNAs being controlled inducibly by one or more stimuli provided at a desired time. This type of alphavirus producer cell line configuration essentially provides a cascade of events that include: inducible production of vector RNA and resulting autocatalytic cytoplasmic amplification of the RNA, the induction of high level structural protein expression by vector-supplied nonstructural proteins, the packaging of vector RNA by the expressed structural proteins, and the release of packaged vector particles. Tightly regulated, inducible expression of vector RNA from the DNA molecule, once producer cell population reaches as desired number, is preferred, due to the potential for low level cytotoxicity of vector replication, or the necessity to control nonstructural protein synthesis, as it relates to the regulation of positive strand versus negative strand vector RNA ratios.

A. Alphavirus DNA Vectors with Single Level Regulation

In certain embodiments of the present invention, a DNA-based alphavirus vector is provided, wherein *in vivo* transcription of an alphavirus vector RNA molecule that is capable of autocatalytic amplification occurs from a promoter which is regulatable by applying a stimulus at a desired time. Such a DNA-based alphavirus vector subsequently may be stably transformed into an alphavirus packaging cell line (PCL) to create an inducible alphavirus producer cell line. The producer cell line configuration described herein, is therefore, a "feed-forward" system in which: 1) a stimulus is applied to the cell, resulting in efficient transcription of alphavirus vector RNA; 2) the vector RNA replicates autocatalytically and produces nonstructural proteins; 3) the nonstructural proteins stimulate amplification of the structural protein expression cassette mRNAs and high level structural protein expression; and 4) the structural proteins interact with the vector RNA and result in the subsequent packaging of recombinant alphavirus particles which are released into the culture media. Any previously described alphavirus PCL, which is stably transformed with one or more inducible alphavirus structural protein expression cassettes, may serve as the parental line with which to derive the producer cell line.

For example, a tetracycline-responsive promoter system (Gossen and Bujard, *Proc. Natl. Acad. Sci.* 89:5547-5551, 1992) may be utilized for inducible transcription of an alphavirus vector RNA, as depicted in Figure 29. In this system, the expression of a tetracycline repressor and HSV-VP16 transactivator domain, as a "fusion" protein (rTA), stimulates *in vivo* transcription of the alphavirus vector RNA by binding specifically to a tetracycline operator sequence (tetO) located immediately adjacent to a minimal "core" promoter (for example, CMV). The binding and transactivation event is reversibly blocked by the presence of tetracycline, and may be "turned on" by removing tetracycline from the culture media. As uninduced basal levels of transcription will vary among different cell types, other different minimal core promoters (for example HSV-tk) may be linked to the tetracycline operator sequences, provided the transcription start site is known, to allow juxtaposition at or in the immediate proximity of alphavirus vector nucleotide 1.

The rTA transactivator is provided by an additional expression cassette also stably transformed into the same cell line; and in certain embodiments, the rTA expression cassette may itself be autoregulatory. The use of an autoregulatory rTA expression cassette circumvents potential toxicity problems associated with constitutive  
5 high level expression of rTA by linking expression to transcriptional control by the same tetO-linked promoter to which rTA itself binds. This type of system creates a negative feedback cycle that ensures very little rTA is produced in the presence of tetracycline, but becomes highly active when the tetracycline is removed (Figure 29). Such an autoregulatory rTA expression cassette is provided in plasmid pTet-tTAk  
10 (Shockett et al., *Proc. Natl. Acad. Sci. USA* 92:6522-6526, 1995).

Functionality of such a tetracycline-regulated DNA-based alphavirus vector is demonstrated by constructing a modified SIN-1-derived luciferase plasmid vector, which is driven by a tetracycline operator/CMV minimal promoter. Using plasmids pBG/SIN-1 ELVS1.5-luc (Example 4) and pBGSV3' (Example 6) as starting  
15 material, an approximately 7200 bp fragment, including much of the SIN-1 nonstructural-encoding region, the junction region promoter and luciferase reporter gene, and a portion of the 3'-UTR, is isolated by digestion of pBG/SIN-1 ELVS1.5-luc with *Bgl II* and *Fsp I*, and purification from a 0.7% agarose gel using GENECLAN II. The 7200 bp fragment is subsequently ligated into plasmid pBGSV3' that has also been  
20 digested with *Bgl II* and *Fsp I*, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting construct is designated pBGSVdIB/SIN1-luc. Insertion of the remaining sequences, which include the heptamerized tetracycline operator and minimal CMV promoter (tetO/CMV) linked to Sindbis nucleotides 1-2289, such that transcription will initiate with one additional  
25 nonviral nucleotide 5' of Sindbis nucleotide 1, is accomplished by overlapping PCR. In PCR reaction #1, the approximately 370 bp tetO/CMV portion of the sequence is amplified by standard three-cycle PCR with a 30 second extension from template plasmid pUHC13-3 (Gossen and Bujard, *ibid*) using the following oligonucleotide primers that are designed to also contain flanking *Bgl II* and *Asc I* sites on one primer  
30 and sequences overlapping 5'-Sindbis nucleotides on the other.

Forward primer: 5'BAtetOF (5'-rest. sites/tetO nts.) (SEQ. ID. NO. 88)  
5'-TATATAGATCTGGCGCGCC/TTTACCACTCCCTATCAGTGATAG-3'

5 Reverse primer: 3'CMVpro/SINR (5'-Sindbis nts./CMV nts.) (SEQ. ID. NO. 89)  
5'-TACGCCGTCAAT/ACGGTTCATAAAGAGCTCTGC-3'

In PCR reaction #2, the 2289 bp Sindbis 5'-end portion of the sequence is amplified by standard three-cycle PCR with a three minute extension, from template plasmid  
10 pKSRSIN-1 (Example 1), using the following oligonucleotide primers that are designed to also contain sequences overlapping the CMV promoter nucleotides on one primer.

Forward primer: CMVSIN5'endF (5'-CMV nts./Sindbis nts.) (SEQ. ID. NO. 90)  
5'-TAGTGAACCGT/ATTGACGGCGTAGTACACACTATT

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Reverse primer: SIN2400R (all Sindbis nts.) (SEQ. ID. NO. 91)  
5'-CGTTGAGCATAACCGAATCTAC

Following amplification, the DNA fragments are purified with QIAquick-spin and used  
20 together as templates in a subsequent three-cycle PCR reaction with 3.5 minute extension, using additional 5'BAtetOF and SIN2400R primers. The resulting overlapping PCR amplicon of approximately 2660 bp is purified using GENECLAN II, digested with Bgl II, and ligated into plasmid pBGSVdIB/SIN1-luc that has also been digested with Bgl II, treated with alkaline phosphatase, and purified from a 0.7%  
25 agarose gel using GENECLAN II. The resulting construct is designated ptetSIN1-luc. Vector constructs containing other heterologous sequences-of-interest are generated using a similar approach, or by direct cloning into the *Xho* I and/or *Not* I sites. Subsequently, a selectable *E. coli gpt* gene (xanthine-guanine phosphoribosyltransferase) expression cassette is generated and inserted into the unique *Pac* I site of  
30 plasmid ptetSIN1-luc, to provide an additional selectable marker. First, a fragment containing the SV40 promoter linked to a *gpt* gene open reading frame is amplified

from plasmid pMAM (Clontech, Palo Alto, CA) by standard three-cycle PCR with a 2 minute extension, and using the following oligonucleotide primers that are designed to contain upstream flanking *Sac* I and *Pac* I sites and a downstream *Sac* I site.

5 Forward primer: SV40proSPF (5'-rest. sites/SV40 promoter seq.) (SEQ. ID. NO. 92)  
5'-ATATAGAGCTCTTAATTAA/TCTTTGTGAAGGAACCTTACTTC

Reverse primer: 3'ECgptR (5'-rest. site/gpt gene seq.) (SEQ. ID. NO. 93)  
5'-ATATAGAGCTC/AGGCGTTGAAAAGATTAGCGACCG

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Following amplification, the SV40 promoter/*gpt* gene DNA fragment is purified with QIAquick-spin, digested with *Sac* I, purified using GENECLAN II, and ligated into plasmid pBGS131 dLXhoI-BGHTT (Example 5) that also had been digested with *Sac* I, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using  
15 GENECLAN II. Clones with proper orientation of the insert are identified by restriction analysis. This configuration positions the promoter and *gpt* gene immediately adjacent to a bovine growth hormone transcription termination signal. The resulting *gpt* expression construct is designated pBGS131 dLXhoI-gpt. Next the entire expression cassette is amplified from plasmid pBGS131 dLXhoI-gpt by standard three-  
20 cycle PCR with a 2 minute extension, and using the following oligonucleotide primers that are designed to contain flanking *Pac* I sites.

Forward primer: SV40proSPF, as shown above (SEQ. ID. NO. 92)

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Reverse primer: BGHTTpacR (5'-rest. site/BGH seq.) (SEQ. ID. NO. 94)  
5'-TATATATTAATTAA/ATAGAATGACACCTACTCAGACAATGCGATGC

Following amplification, the *gpt* gene expression cassette fragment is purified with  
30 QIAquick-spin, digested with *Pac* I, purified using GENECLAN II, and ligated into the tet-inducible alphavirus vector construct ptetSIN1-luc that also had been digested

with *Pac* I, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting construct is designated ptetSINlgpt-luc.

For construction of an initial tetracycline-inducible alphavirus vector producer cell line, the ptetSINlgpt-luc construct and a tetracycline repressor/VP16 transactivator (rTA) expression cassette are stably transformed into the desired alphavirus PCL. For example, alphavirus C/GLYCO PCL cells (from above) are stably transformed with plasmid pTet-tTAk (see above) by cotransfection with another plasmid encoding a selectable marker. Plasmids pTet-tTAk and pSV2-His, encoding a histidinol dehydrogenase marker (Schatz et al., 1989, *Cell* 59:1035-1048), are co-transfected into C/GLYCO PCL cells (or other PCL) at a molar ratio of 40:1, respectively, using Lipofectamine, as described by the manufacturer. Approximately 24 hours post-transfection, the cells are trypsinized and re-plated in media containing histidinol and 0.5 ug/ml tetracycline. The media is exchanged periodically with fresh drug-containing media, and foci of resistant cells are allowed to grow. Cells are trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones are grown are expanded for screening. Positive pTet-tTAk-containing packaging cell clones, designated C/GLYCO/TAk cells, are identified by transfecting the luciferase reporter plasmid pUHC13-3 (Gossen and Bujard, *ibid*), under the control of a tetO/promoter, in both the presence or absence of tetracycline. In the absence of tetracycline, positive C/GLYCO/TAk PCL cells will provide induction from the tetO/promoter and inducible, high levels of luciferase.

Subsequently, the DNA-based alphavirus vector construct ptetSINlgpt-luc is stably transfected into the C/GLYCO/TAk cells using Lipofectamine, as described by the manufacturer. Approximately 24 hr post-transfection, the cells are trypsinized and re-plated in selection media, optimized for the particular cell type (DMEM + 10% dialyzed fetal calf serum; 250 ug/ml xanthine; 15 ug/ml hypoxanthine; 10 ug/ml thymidine; 2 ug/ml aminopterin; 25 ug/ml mycophenolic acid), and containing 0.5 ug/ml tetracycline. The media is exchanged periodically with fresh selection media, and foci of resistant cells are allowed to grow. Cells are trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones are grown



are expanded for screening. Positive producer cell lines, stably transformed with ptetSIN1gpt-luc, are identified by removing tetracycline from the media for at least 24 hr and testing for luciferase in cell lysates and also testing for packaged luciferase vector in the culture supernatants, as described previously.

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#### B. Alphavirus DNA Vectors With Two Level Regulation

In preferred embodiments, it may be desirable to construct a DNA-based alphavirus vector (wild-type or with the desired phenotype of reduced, delayed or no inhibition of host macromolecular synthesis), wherein transcription of the RNA vector molecule, capable of autocatalytic amplification, occurs from a promoter which is very tightly controlled by two levels of regulation to eliminate all basal levels of transcription. Such an approach may combine one inducible component (*e.g.*, the tet system from above) with a reversible transcriptional silencing component. For example, the KRAB repression domain of a certain zinc finger protein may be used.

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Briefly, KRAB (Krüppel-associated box) domains are highly conserved sequences present in the amino-terminal regions of more than one-third of all Krüppel-class Cys<sub>2</sub>His<sub>2</sub> zinc finger proteins. The domains contain two predicted amphipathic  $\alpha$ -helices and have been shown to function as DNA binding-dependent RNA polymerase II transcriptional repressors (for example, Licht et al., *Nature* 346: 76-79, 1990). Like other transcription factors, the active repression domain and the DNA-binding domain are distinct and separable. Therefore, the repression domain can be linked as a fusion protein to any sequence specific DNA binding protein for targeting. Ideally, the DNA binding protein component can be reversibly prevented from binding in a regulatable fashion, thus turning "off" the transcriptional silencing. For example, within one embodiment the KRAB domain from human Kox1 (Thiesen, *New Biol.* 2:363-374, 1990) is fused to the DNA-binding lactose (lac) repressor protein, forming a hybrid transcriptional silencer with reversible, sequence-specific binding to a lac operator sequence engineered immediately adjacent to the tet-responsive promoter (Figure 30). In this configuration, constitutive expression of the lac repressor/KRAB domain fusion (rKR) will result in binding to the lac operator sequence and the

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elimination of any "leaky" basal transcription from the uninduced tet-responsive promoter. When vector expression is desired and tetracycline is removed from the system, IPTG is added to prevent rKR-mediated transcriptional silencing.

In addition, the KRAB domains from other zinc finger proteins, for example, ZNF133 (Tommerup et al., *Hum. Mol. Genet.* 2:1571-1575, 1993), ZNF91 (Bellefroid et al., *EMBO J.* 12:1363-1374, 1993), ZNF2 (Rosati et al., *Nucleic Acids Res.* 19:5661-5667, 1991), and others, as well as other transferable repressor domains, for example, *Drosophila en* or *eve* genes (Jaynes and O'Farrell, *EMBO J.* 10:1427-1433, 1991; Han and Manley, *Genes Dev.* 7:491-503, 1993), human zinc finger protein YY1 (Shi et al., *Cell* 67:377-388, 1991), Wilms' tumor suppressor protein WT1 (Madden et al., *Science* 253:1550-1553, 1991), thyroid hormone receptor (Baniahmad et al., *EMBO J.* 11:1015-1023, 1992), retinoic acid receptor (Baniahmad et al., *ibid*), Kid-1 (Witzgall et al., *Proc. Natl. Acad. Sci. USA* 91:4514-4518, 1994), are readily used in such a system. Furthermore, the lac repressor/lac operator component of this system may be substituted by any number of other regulatable systems derived from other sources, for example, the tryptophan and maltose operons, GAL4, etc.

Specifically, an expression cassette that contains the lac repressor (*lacI*) protein fused to the KRAB domain of human Kox1, with a linked nuclear localization sequence (NLS; Pro-Lys-Lys-Lys-Arg-Lys (SEQ. ID. NO. 100); Kalderon et al., *Cell* 39:499-509, 1984) to more efficiently direct the protein back to the nucleus, is constructed by overlapping PCR. In PCR reaction #1, the approximately 1100 bp *lacI* sequence is amplified by standard three-cycle PCR with a 1.5 minute extension, from template plasmid p3'SS (Stratagene, La Jolla, CA), using the following oligonucleotide primers that are designed to also contain a flanking *Xho* I site and AUG start codon in good translation initiation context on the upstream primer, and the SV40 large-T-antigen nuclear localization sequence on the other.

Forward primer: *LacI*5'F (5'-rest. site/AUG + *lacI* sequence) (SEQ. ID. NO. 95)

5'-ATATACTCGAGTAGCA/ATGGTGAAACCAGTAACGTTATAC

Reverse primer: LacI3'NLSR (5'-NLS/lacI sequence) (SEQ. ID. NO. 96)

5'-GCCCTTTCTCTTCTTTTTTGG/CTGCCCGCTTCCAGTCGGGAAAC

In PCR reaction #2, the an approximately 400 bp amplicon, comprising the amino-terminal 121 residue KRAB domain of human Kox1 is amplified by standard three-cycle PCR with a one minute extension, from template plasmid pKox1 (Thiesen, *New Biol.* 2:363-374, 1990), using the following oligonucleotide primers that are designed to also contain sequences overlapping NLS and lacI on one primer and a *Sac I* restriction site and stop codon on the other.

10

Forward primer: KRAB5'F (5'-NLS+lacI overlap sequence/KRAB sequence)

(SEQ. ID. NO. 97)

5'-CCAAAAAAGAAGAGAAAG/GGCGGTGGTGCTTTGTCTCCT

15 Reverse primer: KRAB3'R (5'-rest. site+stop codon/KRAB sequence)

(SEQ. ID. NO. 98)

5'-ATATAGAGCTCTTA/AACTGATGATTTGATTTCAAATGC

Following amplification, the DNA fragments are purified with QIAquick-spin and used together as templates in a subsequent three-cycle PCR reaction with 2.5 minute extension, using additional LacI5'F and KRAB3'R primers. The resulting overlapping PCR amplicon of approximately 1500 bp is purified using GENECLAN II, digested with *Xho I* and *Sac I*, and ligated into the eukaryotic expression vector plasmid pEUK-C1 (Clontech, Palo Alto, CA) that has also been digested with *Xho I* and *Sac I*, treated with alkaline phosphatase, and purified from a 0.7% agarose gel using GENECLAN II. The resulting *lacI*/KRAB expression construct is designated pEUK-rKR.

To generate stable PCL transformants containing the *lacI*/KRAB expression cassette, an alphavirus PCL which has already been selected for transformation with an rTA tet/transactivator fusion protein cassette is used for starting material. For example, alphavirus C/GLYCO/Tak PCL cells (from above) are stably

30

transformed with plasmid pEUK-rKR by cotransfection with another plasmid encoding a selectable marker. Plasmids pEUK-rKR and pPUR, encoding a puromycin acetyltransferase selectable marker (Clontech), are co-transfected into C/GLYCO/Tak PCL cells (or other PCL) at a molar ratio of 40:1, respectively, using Lipofectamine, as  
5 described by the manufacturer. Approximately 24 hr post-transfection, the cells are trypsinized and re-plated in media containing 5 ug/ml puromycin and 0.5 ug/ml tetracycline. The media is exchanged periodically with fresh drug-containing media, and foci of resistant cells are allowed to grow. Cells are trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones are grown  
10 are expanded for screening. Positive pEUK-rKR-containing packaging cell clones, designated C/G/Tak/rKR cells, are identified by immunostaining with a polyclonal antiserum specific for *lacI* (Stratagene, La Jolla, CA).

Next, specific lac operator (*lacO*) sequences must be inserted into the desired ptet-based alphavirus vector (see above). For example, vector construct  
15 ptetSINlgpt-luc is modified to contain multiple copies of *lacO* by using a synthetic oligonucleotide linker. The *LacO* oligonucleotide is designed to contain a symmetric *lacO* sequence, including the full 22 bp palindromic operator sequence (Simons et al., *Proc. Natl. Acad. Sci. USA* 81:1624-1628, 1984; Sadler et al., *Proc. Natl. Acad. Sci. USA* 80:6785-6789, 1983), and flanking *Asc* I sites when self-annealed into a double-  
20 stranded molecule.

*LacOsymA* (SEQ. ID. NO. 99)

5'-CGCGCCGAATTGTGAGCGCTCACAATTCGG

25 The *LacOsymA* oligo is self-annealed to form a *Asc* I "sticky-ended" DNA fragment, and then ligated into plasmid ptetSINlgpt-luc that has been digested with *Asc* I, treated with alkaline phosphatase, and purified from a 0.7% gel using GENECLAN II. Clones containing one, two, three, or more tandem copies of the *lacO* sequence are identified by sequence analysis, and given the designation pOIItetSINlgpt-luc,  
30 pOIIItetSINlgpt-luc, pOIIIItetSINlgpt-luc, etc. Individual clones with different *lacO*

copy numbers are then transfected as detailed below, and tested for the tightest level of transcriptional regulation.

To generate an alphavirus vector producer cell line, the DNA-based pOtetSIN1gpt-luc vector constructs are stably transfected into C/G/Tak/rKR cells using  
5 Lipofectamine, as described by the manufacturer. Approximately 24 hr post-transfection, the cells are trypsinized and re-plated in selection media, optimized for the particular cell type (DMEM + 10% dialyzed fetal calf serum; 250 ug/ml xanthine; 15 ug/ml hypoxanthine; 10 ug/ml thymidine; 2 ug/ml aminopterin; 25 ug/ml mycophenolic acid), and containing 0.5 ug/ml tetracycline. The media is exchanged periodically with  
10 fresh selection media, and foci of resistant cells are allowed to grow. Cells are trypsinized and cloned by limiting dilution in 96 well tissue culture dishes, and individual cell clones are grown and expanded for screening. Positive producer cell lines, stably transformed with the pOtetSIN1gpt-luc constructs, are identified by the expression of luciferase (described previously) at least 24 hr after the removal of  
15 tetracycline from the media and the addition of 20mM IPTG for induction. Luciferase activity is determined both on producer cell lysates and also after transfer-of-expression experiments using culture supernatants.

Additional levels of control may be incorporated by adding a third, or even fourth, level of regulation to the promoter responsible for transcription of the  
20 alphavirus vector molecule. Such extra level or regulation may be incorporated into the minimal promoter, and may involve other inducible systems and/or cell differentiation control. In each of the above cases, stable transformation may be accomplished as an integration into the host cell chromosome, or as an extrachromosomal episome, using for example, the EBV episomal-based vector promoter (for non-integrated).

EXAMPLE 8METHODS FOR THE GENERATION OF ALPHAVIRUS-DERIVED EMPTY  
OR CHIMERIC VIRAL PARTICLES

5 As illustrated in Example 6, individual defective helper (DH) expression cassettes can be constructed to contain elements from multiple alphaviruses or their variants. Thus, as described in Example 6, split structural gene DH cassettes for the expression of the viral glycoproteins can be constructed to contain the capsid and glycoprotein genes from different alphavirus species. For example, such a heterologous  
10 alphavirus glycoprotein DH cassette might contain the capsid gene from Ross River virus (RRV), and the glycoprotein genes from Sindbis virus. In this configuration, the RRV capsid gene serves to enhance the level of translation of the glycoprotein genes.

The configurations described herein for the heterologous alphavirus glycoprotein DH cassettes are designed to improve the packaging of vector replicons  
15 into alphavirus particles, yet diminish the possibility of recombination, resulting in the formation of replication competent alphavirus. The heterologous alphavirus glycoprotein DH expression cassette is a replacement of the Sindbis virus capsid gene in the DH expression cassettes described in Example 6 ("genomic" structural protein gene PCL), with a heterologous alphavirus capsid gene (e.g. RRV). The second DH  
20 expression cassette in the split structural gene PCL contains, for example, the Sindbis virus capsid gene. Thus, a split structural gene PCL for the generation of recombinant alphavirus vector particles having Sindbis virus structural proteins can be derived, for example, with the Sindbis virus glycoprotein genes and capsid genes on individual DH expression cassettes.

25 It has been shown previously that chimeric viruses containing all of the genes of RRV, but with the capsid gene from Sindbis virus, or the reciprocal chimeric virus, do not assemble into infectious virus particles (Lopez et. al., *J. Virol.* 68:1316-1323, 1994). The authors concluded in this report that the interaction between the carboxy terminus of glycoprotein E2 and capsid protein in virus assembly cannot occur  
30 between the structural proteins of heterologous alphaviruses. Thus, recombinant

genomes arising in the split structural gene alphavirus PCLs described in Example 6, consisting of the Sindbis virus non-structural protein genes (originating from the vector replicon), the RRV capsid gene, and the Sindbis virus glycoprotein genes, should not be replication competent, resulting in the propagation of virus (replication competent  
5 Sindbis virus, RCSV). The packaging restriction between heterologous alphavirus species permits the construction of DH cassettes comprised of the capsid gene, including the translational enhancement element, from one alphavirus, and the glycoprotein genes from a different alphavirus.

However, as illustrated in Figure 31, the observation of Lopez et. al.  
10 (*ibid*), that assembly cannot occur between the structural proteins of heterologous alphaviruses, is incorrect. Indeed, a DH cassette consisting of the RRV capsid gene, and the Sindbis virus glycoprotein gene produces infectious virus particles. Briefly, BHK cells were co-electroporated with SINrep/Lac Z replicon (Bredenbeek et. al., *J. Virol.*, 67:6439-6446, 1993), and DH-BB (5' tRNA/SIN) Crrv (Example 6 and Figure  
15 24; RRV capsid/Sindbis virus glycoproteins) *in vitro* transcribed RNAs. The electroporation and *in vitro* transcriptions were performed as described in Example 1. Following electroporation, the BHK cells were treated with dactinomycin and labeled with [<sup>3</sup>H]uridine, exactly as described in Example 1. At 18 hours post electroporation, the culture medium was collected, and clarified by centrifugation at 6,000 rpm for 10  
20 min. The vector particles remaining in the supernatant were pelleted by ultracentrifugation, after first layering over a sucrose cushion. RNA was isolated from the BHK cells at 18 hours post electroporation, and from the virus pellet, electrophoresed on denaturing glyoxal agarose gels, and visualized by autoradiography, exactly as described in Example 1. The viral RNAs present in BHK cells electroporated  
25 with SINrep/LacZ and DH-BB Crrv RNAs, and in virus particles, are shown in Figure 31 (lane 1, panel A, and lane 1, panel B). RNAs corresponding to the genomic and subgenomic replicative species for SINrep/LacZ and DH-BB Crrv RNAs were present in both electroporated BHK cells, and the produced virus particles. The results demonstrate, in contrast to Lopez et. al. (*ibid*), the formation of chimeric alphavirus  
30 particles consisting of RRV capsid protein and Sindbis virus glycoproteins. Further, the

indiscriminate packaging of genomic and subgenomic SINrep/LacZ and DH-BB Crrv RNAs in chimeric alphavirus particles indicates the inability of the Ross River capsid protein to recognize specifically the Sindbis virus packaging sequence, which is present in the nsP1 gene of the SINrep/LacZ vector replicon.

5                   The viral proteins present in BHK cells electroporated with SINrep/LacZ and DH-BB Crrv RNAs, at 18 hours post electroporation, and the produced virus particles are given in Figure 32 (lane 1, panel A, and lane 1, panel B). The viral-specific structural proteins in electroporated cells and the produced chimeric alphavirus particles were indistinguishable. That is, Figure 32 demonstrates clearly that virus particles  
10                   produced from BHK cells electroporated with SINrep/LacZ and DH-BB Crrv RNAs contained the RRV capsid and the Sindbis virus glycoproteins E1 and E2. This result provides indisputable evidence that in contrast to Lopez et. al., (*ibid*), there is no restriction in assembly between heterologous alphavirus capsid and glycoproteins that prevents the formation of chimeric viral particles.

15                   Thus, in distinct contrast to the results and discussion of Lopez et. al., the amino terminus of the RRV capsid protein is able to bind with the heterologous Sindbis virus genome, and form infectious chimeric alphavirus particles. Importantly, the previous conclusion that there is a restriction of virus assembly between heterologous alphavirus capsid proteins and glycoproteins is incorrect. The generation of chimeric  
20                   alphavirus particles as described here would, then, also result in the formation of RCSV in the split structural gene PCLs described above, since a recombinant genome consisting of the Sindbis virus non-structural protein genes (originating from the vector replicon), the RRV capsid gene, and the Sindbis virus glycoprotein genes, would generate infectious virus. Alternatively, this lack of restriction of packaging between  
25                   distinct alphavirus structural proteins and vector replicons permits the tropism of vector particles to be modified. For example, Sindbis virus replicons can be packaged with the Venezuelan Equine Encephalitis virus structural proteins, in order to generate a lymphotropic recombinant vector particle.

                  The results described in two separate previous investigations have shown  
30                   that ablation, *in vitro*, of the interaction between the capsid protein and the positive



RNA-stranded genome of two icosahedral viruses having triangulation numbers (T)=3, turnip crinkle virus (TCV), and southern bean mosaic virus (SBMV), resulted in the disassociation of the virus particles, and the formation of nucleic acid-free T=1 particles (Sorger et. al. J. Mol. Biol., 191:639-656, 1986, and Erickson and Rossmann, Virology 5 116:128-136, 1982). In the absence of nucleic acid, T=3 particles similar to wild-type virus were not formed *in vitro*. Owen and Kuhn (J. Virol., 70:2757-2763, 1996), investigated the packaging properties of Sindbis virus genomes containing deletions in the capsid, in order to identify the region of the capsid protein that is required for dictating specificity of the encapsidation reaction, *in vivo*. One mutant virus [CD(97-10 106)] which contained a deletion corresponding to residues 97-106 of the capsid, encapsidated both genomic and subgenomic RNAs, indicating the domain of the capsid protein required for specific recognition of the genomic RNA packaging signal. In yet another report, the packaging properties of Aura alphavirus were investigated (Rumenapf et. al. J. Virol., 69:1741-1746, 1995). In this study, a mechanism for 15 alphavirus packaging that involves a capsid protein-encapsidation sequence interaction initiation complex was proposed. This mechanism proposed is based on observations by the authors, and others (including Owen and Kuhn, *ibid*), in which 26S and 49S alphavirus RNAs are packaged into T=1, T=3, T=4, and T=7 virus particles, and that empty capsids arising during infection with alphaviruses have not been reported.

20               Based on the literature presented above and the discussions contained therein, a RRV capsid gene deleted of the region corresponding to the capsid protein domain that is required for dictating specificity of the encapsidation reaction, and, in addition, surrounding basic residues that bind electrostatically with viral RNA, should not be able to form stable capsid particles containing viral RNAs. Thus, the alphavirus 25 structural proteins expressed from a heterologous alphavirus DH cassette, consisting of this deleted RRV capsid gene and the Sindbis virus glycoprotein genes, should not assemble into stable chimeric alphavirus particles. Thus, in the split structural gene PCL discussed above and in Example 6, a recombinant genome consisting of the Sindbis virus non-structural protein genes, the RRV capsid gene (deleted of the region 30 corresponding to packaging specificity and the surrounding basic residues), and the

Sindbis virus glycoprotein genes, could not generate infectious virus. As described in Example 6, the Sindbis virus capsid protein is expressed from a separate DH expression cassette; thus, the three Sindbis virus structural proteins are expressed in toto, resulting in the production of recombinant vector particles.

5 Nucleotides of the RRV capsid gene corresponding to predicted regions of the expressed protein that bind to the Sindbis virus packaging sequence (Weiss et. al., Nuc. Acids, Res., 22:780-786, 1994, and Lopez et. al., *ibid*), including the basic residues which bind electrostatically with the viral RNA, were deleted in order to construct a heterologous alphavirus capsid-glycoprotein DH that provided translational  
10 enhancement and correct pE2-6K-E1 polyprotein processing by post-translational cleavage, yet could not assemble stable chimeric RRV/Sindbis virus particles. Figure 33 illustrates the hydrophobicity profiles (Kyte-Doolittle) of the RRV capsid protein, and the capsid protein expressed from 3 individual RRV capsid gene mutants (CΔ1rrv, CΔ2rrv, and CΔ3rrv), in which varying amounts of the capsid gene encoding a lysine-  
15 rich protein that interacts with the viral packaging sequence RNA, was deleted. The lysine-rich basic region of the RRV capsid protein is shown in Figure 33. Further, the hydrophobicity profiles demonstrate that this lysine-rich basic region is progressively eliminated in the 3 individual RRV capsid gene mutants CΔ1rrv, CΔ2rrv, and CΔ3rrv. Figure 34 demonstrates the lysine residues eliminated in the expressed RRV capsid  
20 protein, as a result of the deletions in mutants CΔ1rrv, CΔ2rrv, and CΔ3rrv. The table shown below gives the nucleotides deleted in the RRV genome of constructs CΔ1rrv, CΔ2rrv, and CΔ3rrv.

<u>Construct</u>	<u>Deleted RRV genome nts.</u>
CΔ1rrv	7841-7891
CΔ2rrv	7796-7891
CΔ3rrv	7760-7891

25 The RRV capsid gene deletions were constructed on the DH-BB Crrv plasmid DNA illustrated in Figure 23, and described in Example 6. The indicated RRV capsid gene

sequences were deleted by PCR, using the primers and other cloning steps given in Example 6.

Figures 31 and 32, discussed above, illustrate the virus-specific RNAs (Figure 31) and proteins (Figure 32) synthesized in BHK cells electroporated with SINrep/LacZ and the DH-BB CΔ1rrv, CΔ2rrv, or CΔ3rrv RNAs, and present in viral particles contained in the culture fluids of these cells. The genomic and subgenomic species were detected for both the SINrep/LacZ replicon and all the three DH-BB CΔ1rrv, CΔ2rrv, or CΔ3rrv DH RNAs in electroporated cells (Figure 31, panel A). However, the SINrep/lacZ replicon was not packaged in vector particles in cells electroporated with DH RNA containing deletions in the RRV capsid gene, as demonstrated by the absence of replicon genomic RNA in virus particles (Figure 31, panel B). Further, helper genomic and subgenomic RNAs were packaged very inefficiently and were barely visible in autoradiograms of denaturing gels (Figure 31, panel B, lanes 3 and 4), when cells were electroporated with DH molecules containing larger deletions of the RRV capsid (CΔ2rrv or CΔ3rrv). In contrast, while SINrep/lacZ genomic RNA (and DH RNA in electroporations with CΔ2rrv or CΔ3rrv) was not detected in viral particles from BHK cells electroporated with the DHs containing deletions in the RRV capsid gene, equivalent RRV capsid protein and Sindbis virus glycoprotein levels were observed in virus particles from cells electroporated with all DH RNAs, regardless of whether the RRV capsid gene contained deletions (Figure 32, panels A and B). This result demonstrates that stable chimeric virus particles not containing vector replicon, or other viral-specific RNAs, were formed in BHK cells electroporated with SINrep/lacZ genomic RNA and DH RNA, from which capsid protein unable to bind to the genomic RNA was expressed. The formation of stable empty heterologous alphavirus particles is unexpected and not predicted, based on the results and discussions of previous investigations (Lopez et. al., *J. Virol.* 68:1316-1323, 1994, Sorger et. al. *J. Mol. Biol.*, 191:639-656, 1986, Erickson and Rossmann, *Virology* 116:128-136, 1982, and Rumenapf et. al. *J. Virol.*, 69:1741-1746, 1995).

To determine the composition of the virus particles produced, BHK cells were electroporated with SINrep lacZ and the DH RNAs containing various RRV

capsid gene configurations, as described herein, and were treated subsequently with dactinomycin, and labeled with [<sup>35</sup>S]methionine and [<sup>3</sup>H]uridine, as described in Example 1. The configuration of the viral particles produced were determined by ultracentrifugation of clarified cell culture media for 2 hrs at 35,000 rpm in a SW-41 rotor over a 20%-40% (w/w) sucrose gradient. The results of this study are shown in Figures 35-37, and demonstrate again the formation of stable empty heterologous alphavirus particles. Figure 35 demonstrates the relative levels of [<sup>35</sup>S]methionine and [<sup>3</sup>H]uridine incorporated into particles synthesized in BHK cells infected at high MOI (5) with wild-type virus, Toto1101. Figure 36 demonstrates that the relative levels of [<sup>35</sup>S]methionine and [<sup>3</sup>H]uridine incorporated into particles synthesized in BHK cells electroporated with SINrep/LacZ and DH-BB (5' tRNA) Crrv (Figure 23) RNAs was the same as in cells infected with wild-type virus. In contrast, Figure 37 demonstrates that the particles produced in BHK cells electroporated with SINrep/LacZ and DH-BB (5' tRNA) CΔ3rrv contained very low levels of incorporated [<sup>3</sup>H]uridine. Figure 38 is a compilation of Figures 35-37, and illustrates clearly that while the relative levels of [<sup>35</sup>S]methionine and [<sup>3</sup>H]uridine incorporated into particles were similar in BHK cells infected or electroporated with RNAs containing wild-type alphavirus capsid genes, BHK cells electroporated with DH RNA containing deletions of nts. 7760-7891 of the RRV capsid gene formed stable chimeric empty alphavirus particles, devoid of SINrep/LacZ RNA. Titers of empty alphavirus particles, produced in cell lines electroporated with with SINrep/LacZ RNA and DH-BB CD3rrv *in vitro* transcribed RNAs, and labeled with [<sup>35</sup>S]methionine, were determined by comparison with BHK cells, infected with Toto 1101 wild-type virus, and labeled with [<sup>35</sup>S]methionine. The level of radioactivity present in virus-containing sucrose gradient fractions from Toto 1101-infected cells was quantitated, and related to the virus titer present in these same fractions, as determined by plaque assay, according to the methods described in Example 1. For empty alphavirus particle titer determinations, the level of radioactivity present in virus particle-containing sucrose gradient fractions from BHK cells electroporated with SINrep/LacZ RNA and DH-BB CD3rrv *in vitro* transcribed RNAs, was quantitated, and related to the [<sup>35</sup>S]methionine/virus titer, from Toto 1101 infected

cells. The titer of empty chimeric virus particles, containing the deleted Ross River virus capsid and the Sindbis virus glycoproteins, produced in SINrep/LacZ RNA and DH-BB CD3rrv *in vitro* transcribed RNA electroporated cells was  $1 \times 10^9$  particles/ml.

5                   While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art in light of the description, *supra*. Therefore, it is intended that the appended claims cover all such variations coming within the scope of the invention as claimed.

10                   Additionally, the publications and other materials cited to illuminate the background of the invention, and in particular, to provide additional details concerning its practice as described in the detailed description and examples, are hereby incorporated by reference in their entirety.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANTS: Chiron Viagene, Inc.  
Washington University

(ii) TITLE OF INVENTION: RECOMBINANT ALPHAVIRUS-BASED VECTORS  
WITH REDUCED INHIBITION OF CELLULAR MACROMOLECULAR  
SYNTHESIS

(iii) NUMBER OF SEQUENCES: 118

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(E) COUNTRY: USA  
(F) ZIP: 98104-7092

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE: 04-APR-1997  
(C) CLASSIFICATION:

## (vii) ATTORNEY/AGENT INFORMATION:

(A) NAME: McMasters, David D.  
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(C) REFERENCE/DOCKET NUMBER: 930049.457PC

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(A) TELEPHONE: (206) 622-4900  
(B) TELEFAX: (206) 682-6031

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCTCTACGG TGGTCCTAAA TAGT

24

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGTGGAGCTC TAATACGACT CACTATAGAT TGACGGCGTA GTACACAC

48

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AATTTCTGCC TCAGC

15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATGCAAAGT TACTGAC

17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGTCATTAC TTCATGTC

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGTGGATCA CTTTC

15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATTGCGTGAT TTCGTCCGT

19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TAAATTGAG CTTTG

15

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCATATGGC ATTAGTTG

18

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTGGCCATGG AAGGAAAGG

19

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 58 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCCTGAGG GTTTTTTTTT TTTTTTTTTT TTGAAATGTT AAAACAAAA TTTTGTG

58

(2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATATGGGCC CGATTAGGT GAACTATAG ATTGACGGCG TAGTACAC

48

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTGGCAACCG GTAAGTACGA TAC

23

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATACTAGCCA CGGCCGGTAT C

21

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCCTCTTTCG ACGTGTGAG C

21

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACCTTGAGC GCAATGTCCT G

21

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

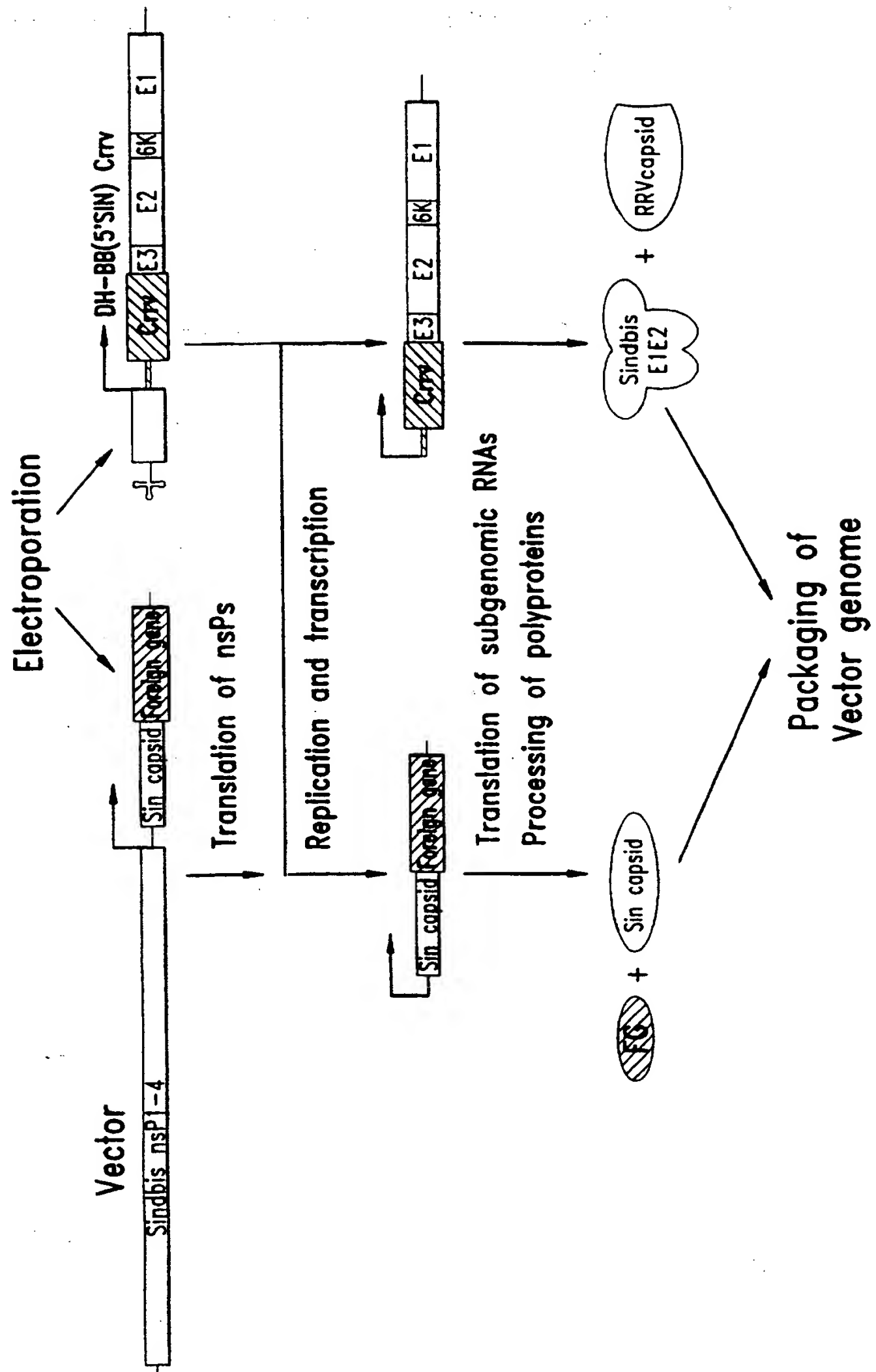


Fig. 24

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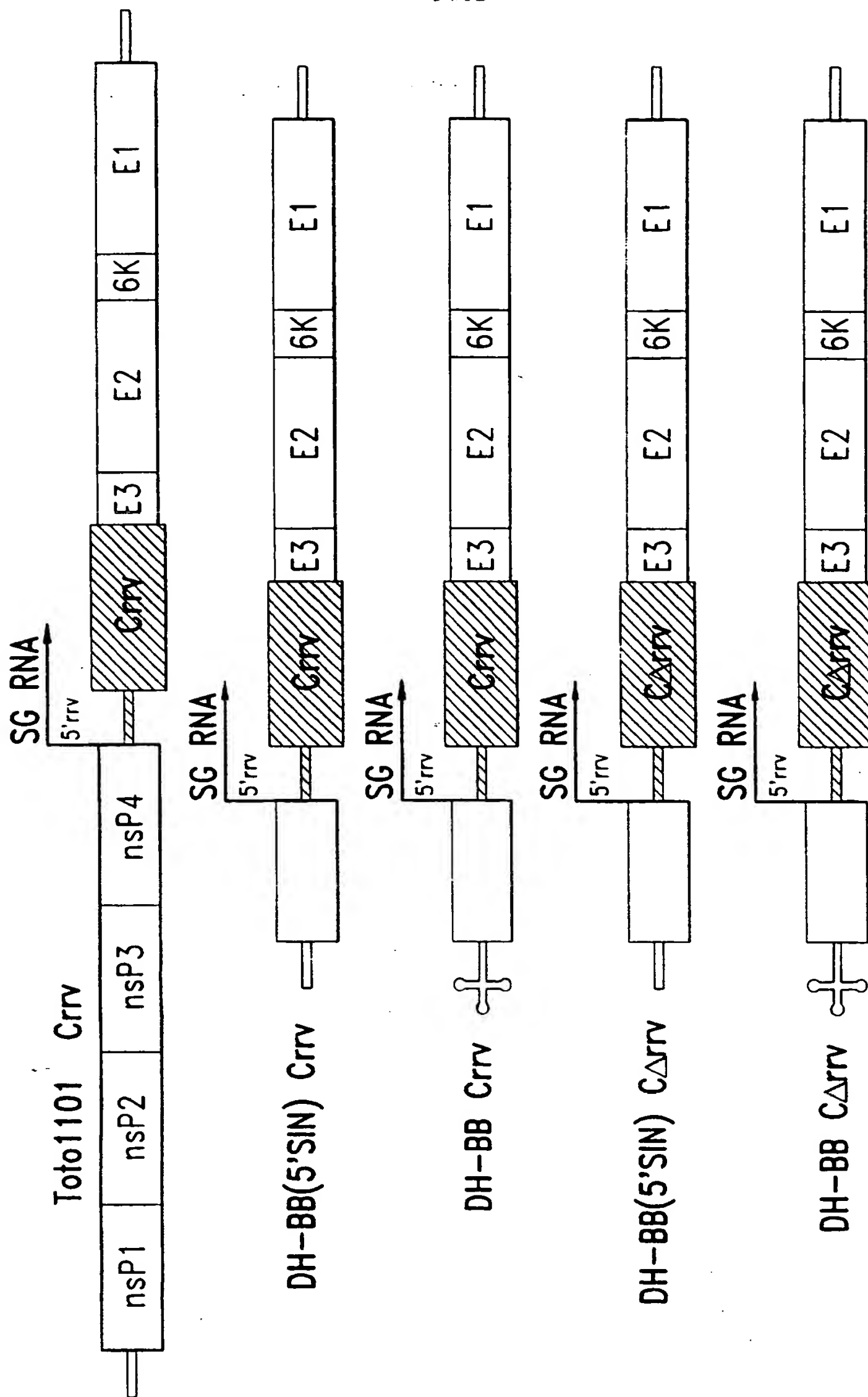


Fig. 23

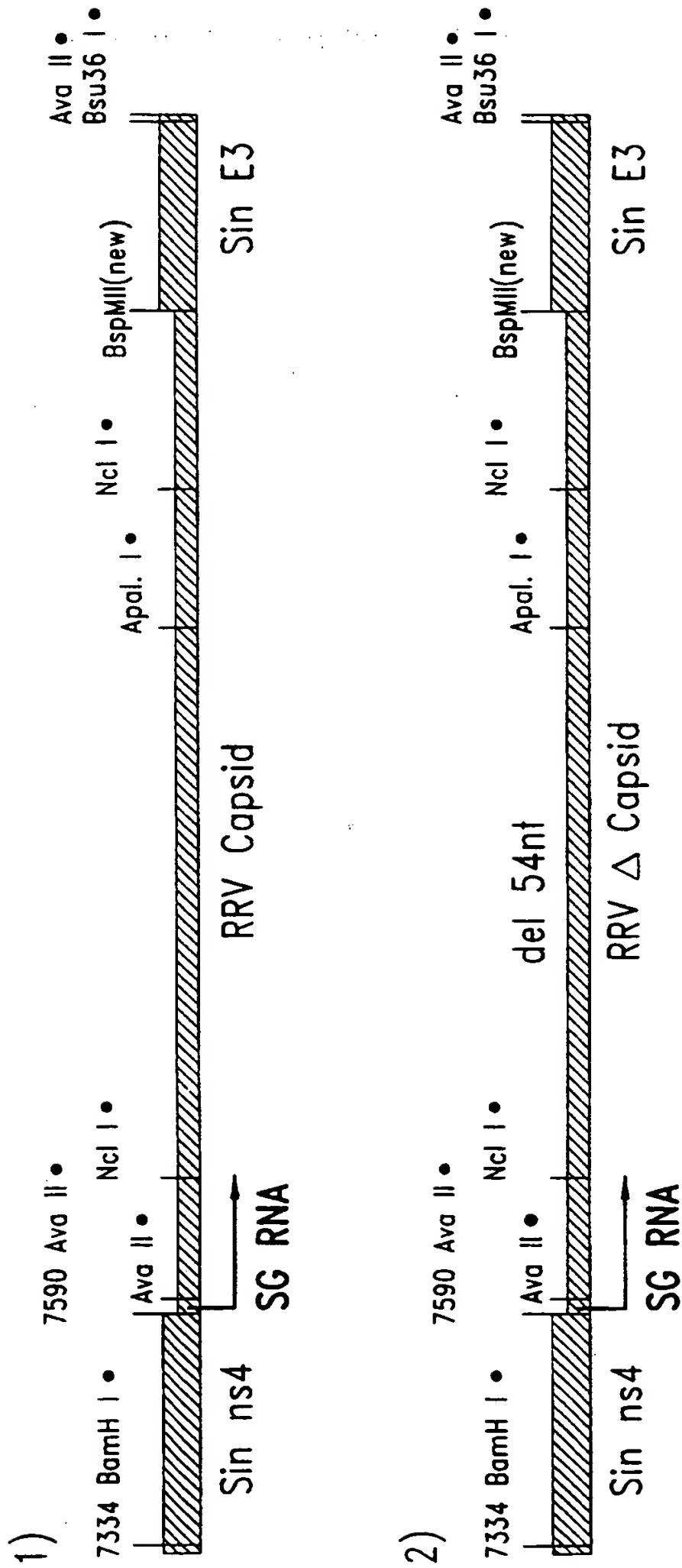
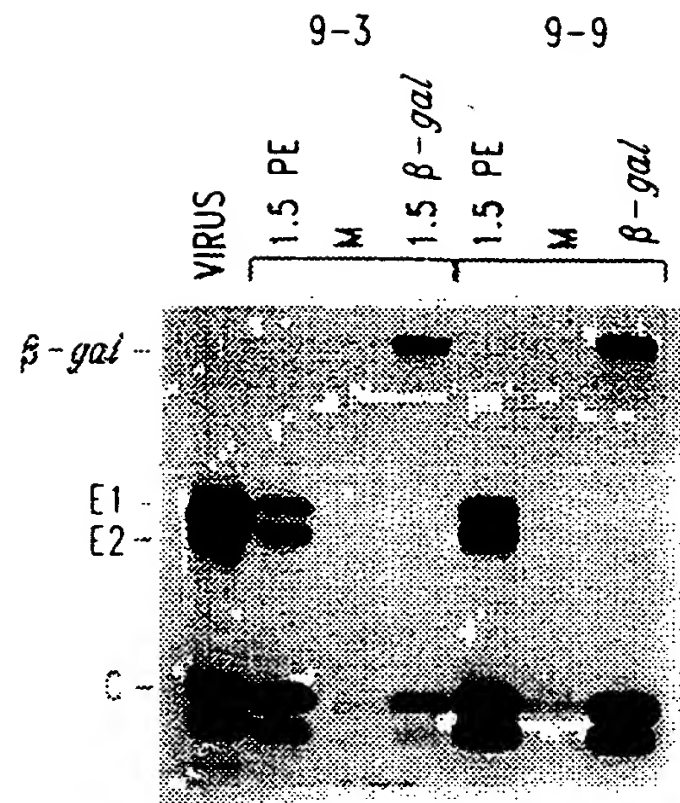


Fig. 22

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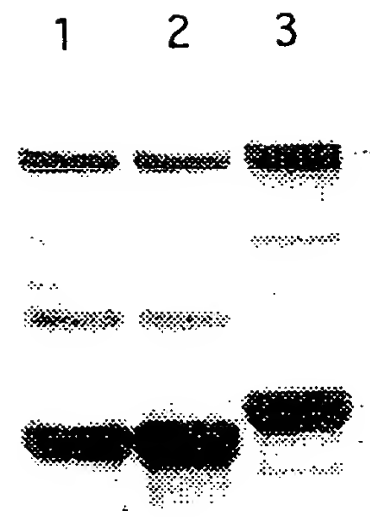
*Fig. 21*



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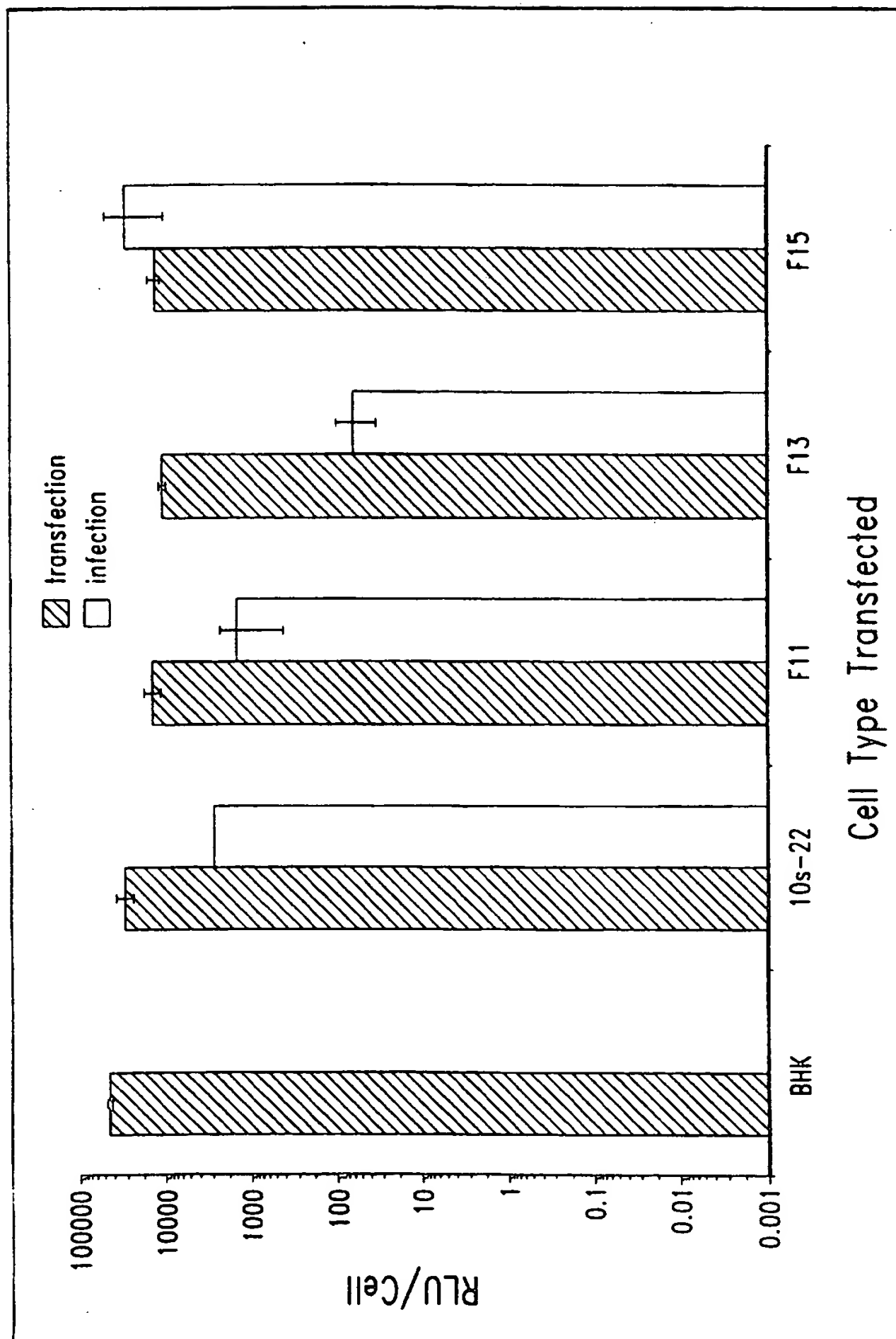
*Fig. 20*

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*Fig. 19*

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SNBST<sup>TM</sup> - Luciferase Vector Packaging by  
pDCMV-intSINrbz and pBGSVCMVdneo Derived PCL



Cell Type Transfected

Fig. 18

Sindbis-luciferase Vector Packaging by  
pBGSVCMVdIneo/Human 293 Packaging Cells

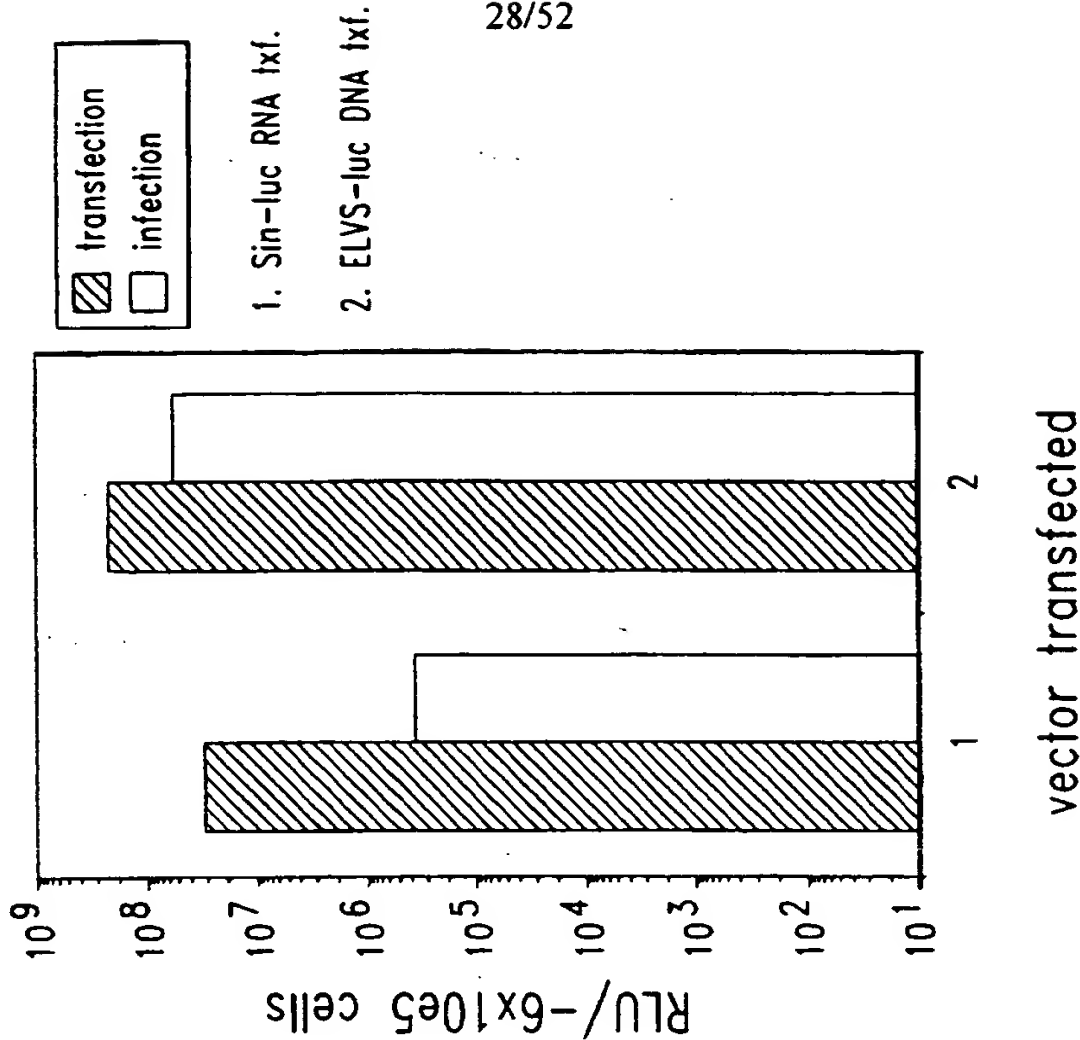


Fig. 17B

Sindbis-luciferase Vector Packaging in  
transfected C6/36 Packaging Cells

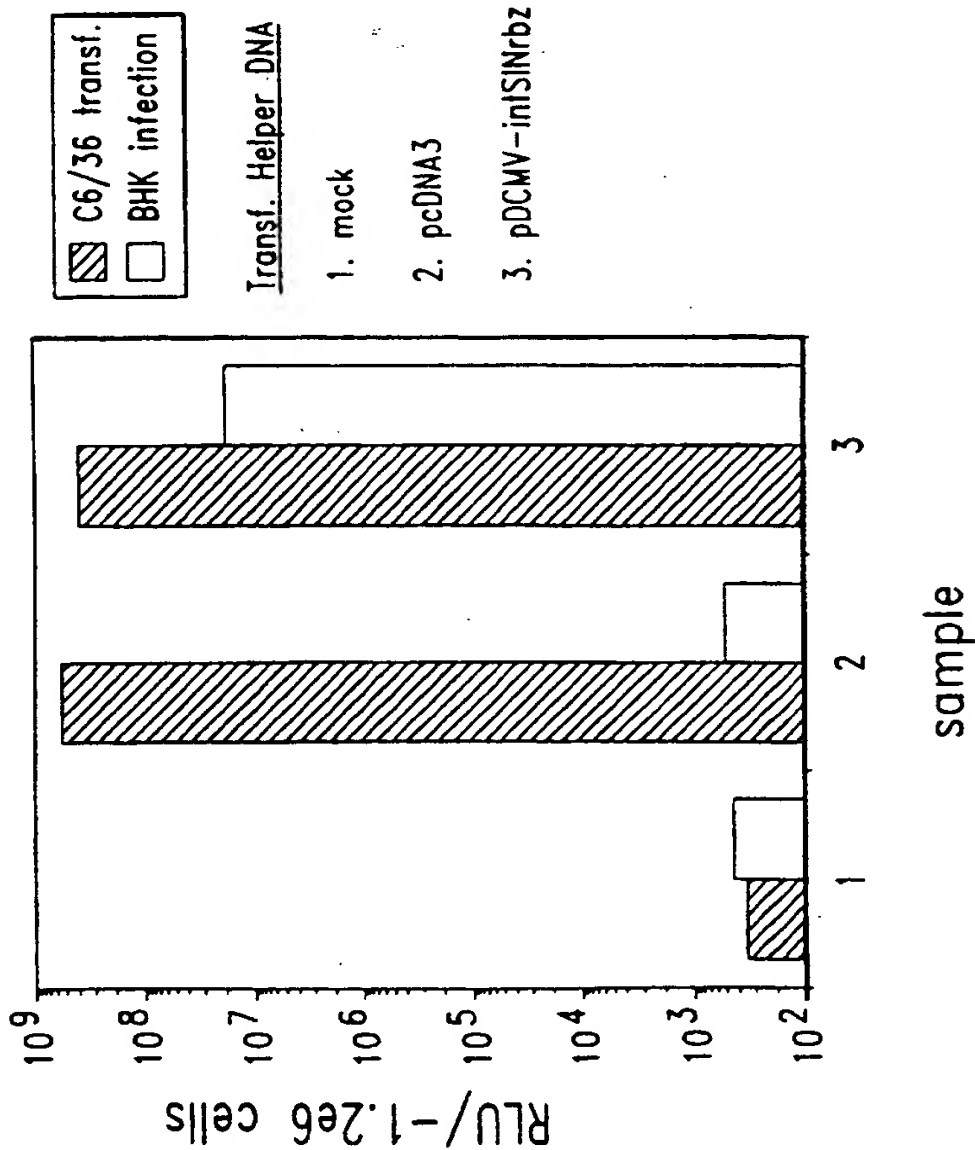
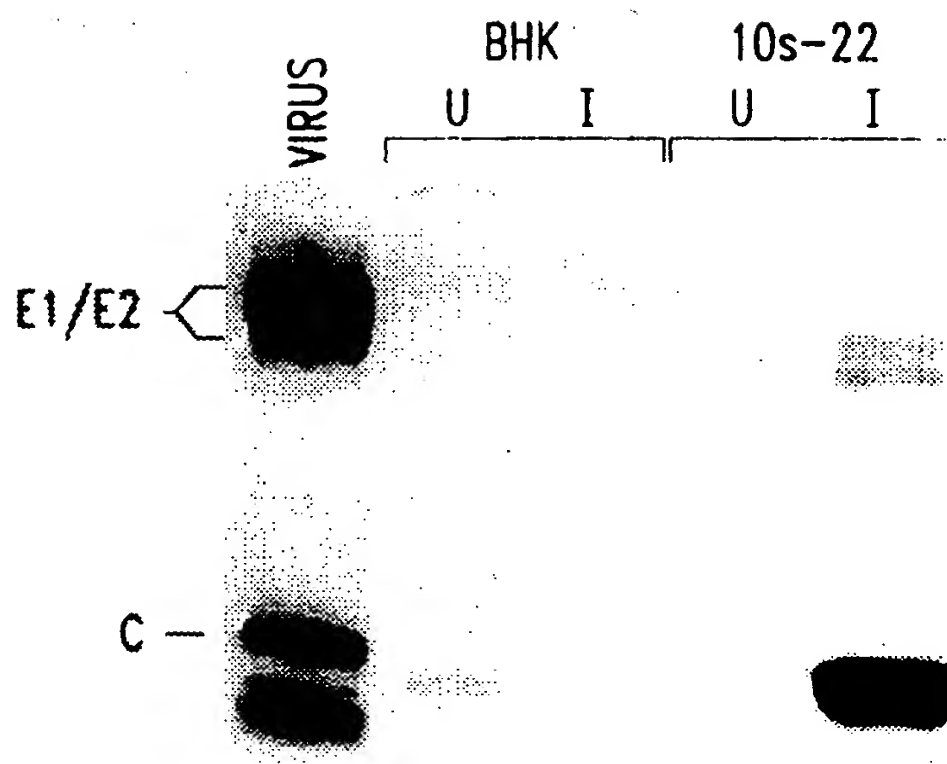


Fig. 17A

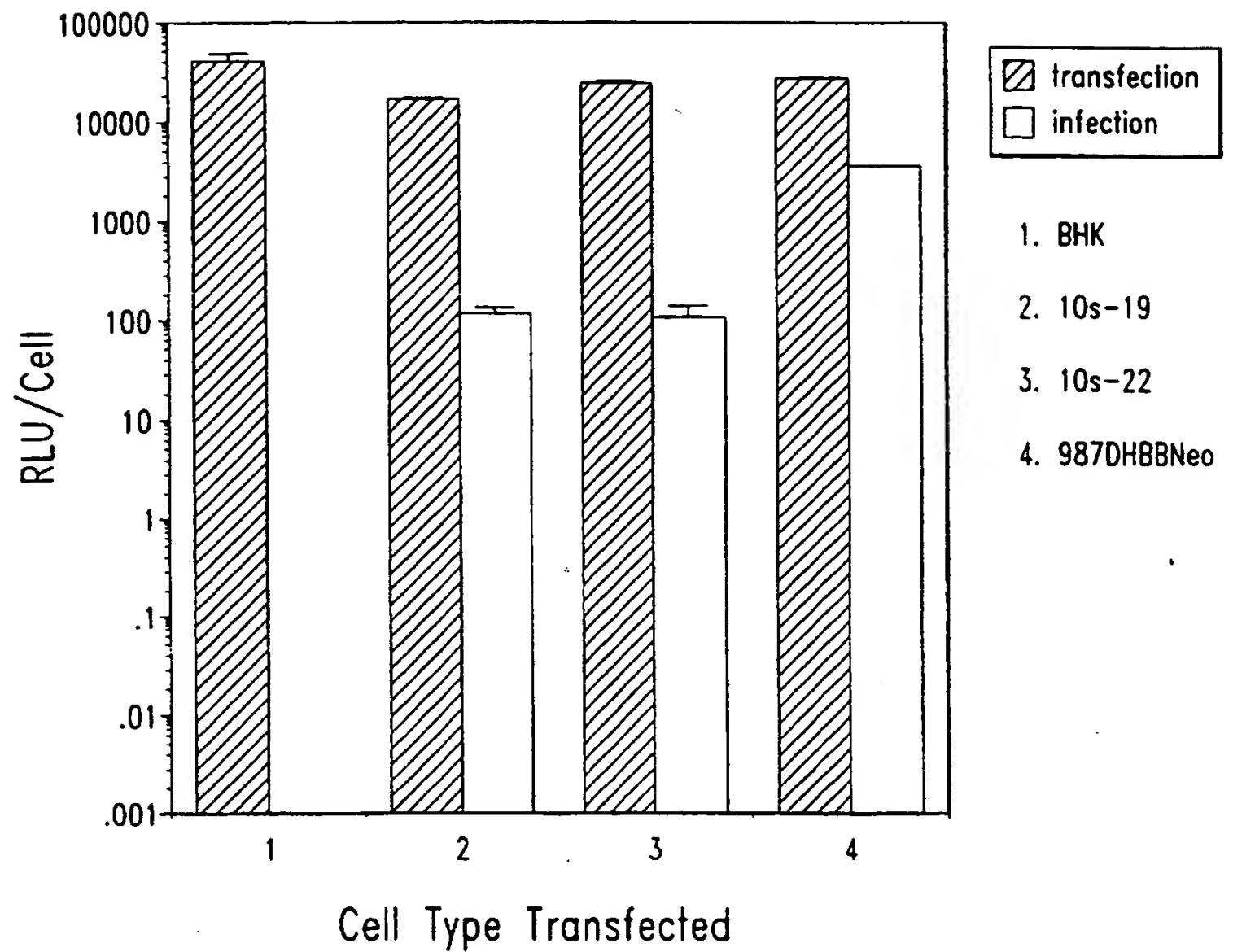
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*Fig. 16*

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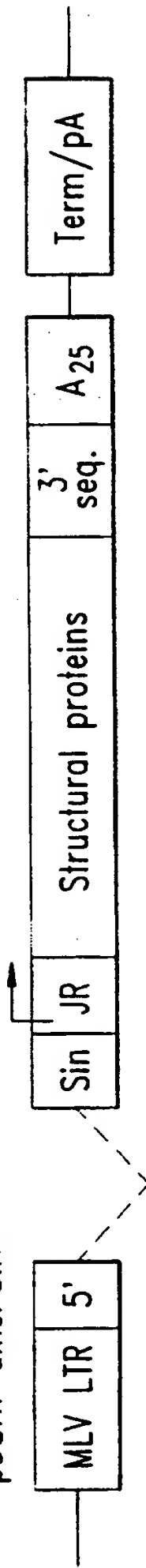
SNBS™-Luciferase Vector Packaging by  
pDCMV-intSINrbz and 987BB Derived PCL



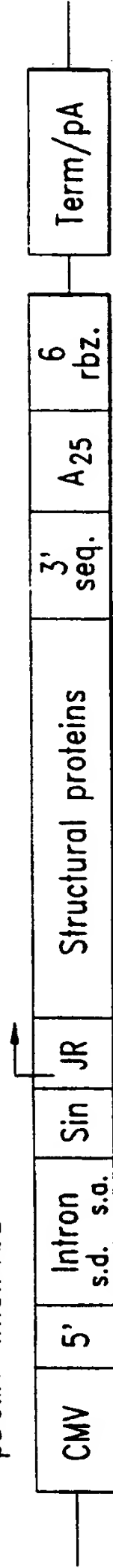
*Fig. 15*

# SNBS™ PACKAGING CELL LINE EXPRESSION CASSETTES

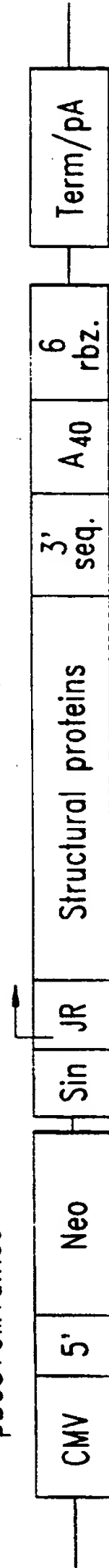
pDLTR-dInsPSIN



pDCMV-intSINrbz



pBGSCVMVdIneo



987DHBBNeo

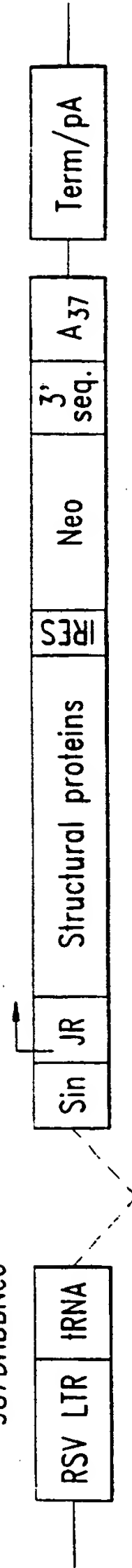


Fig. 14

# Packaging of SNBS™ Vectors by Inducible Packaging Cell Lines

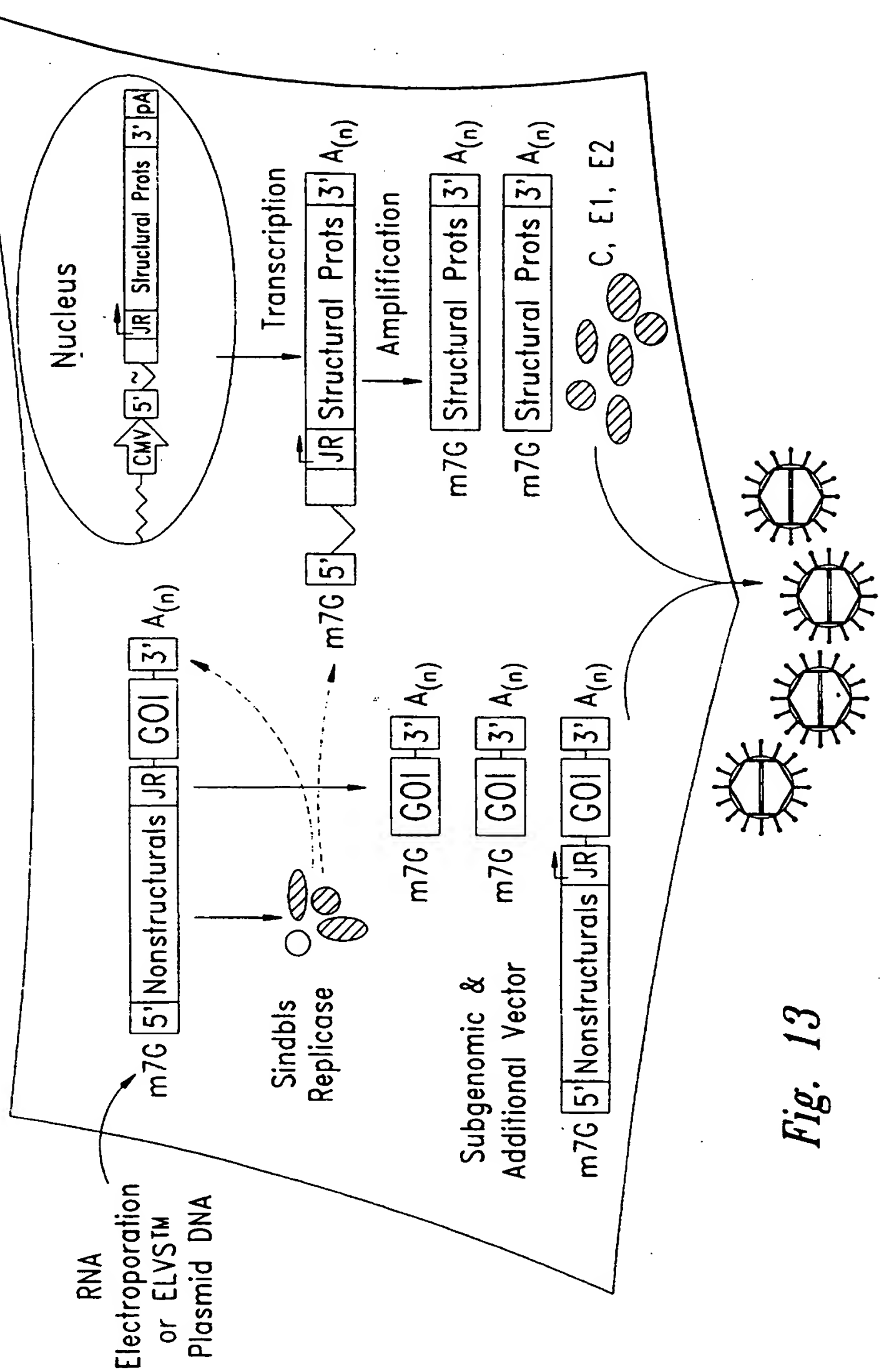
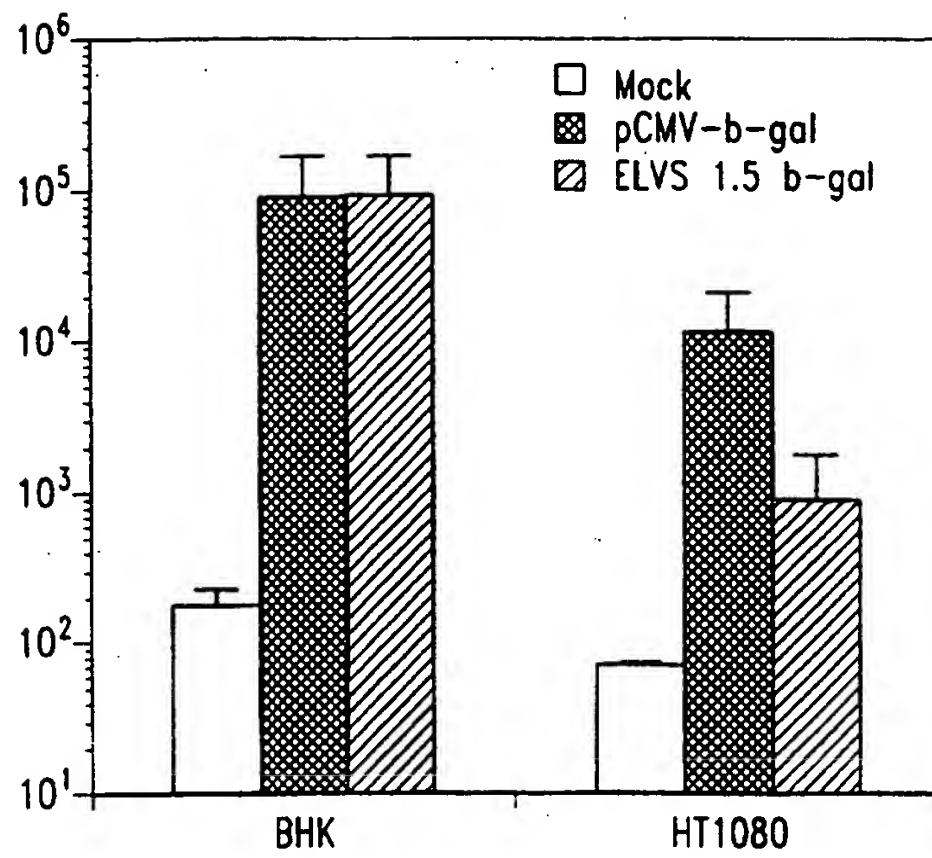
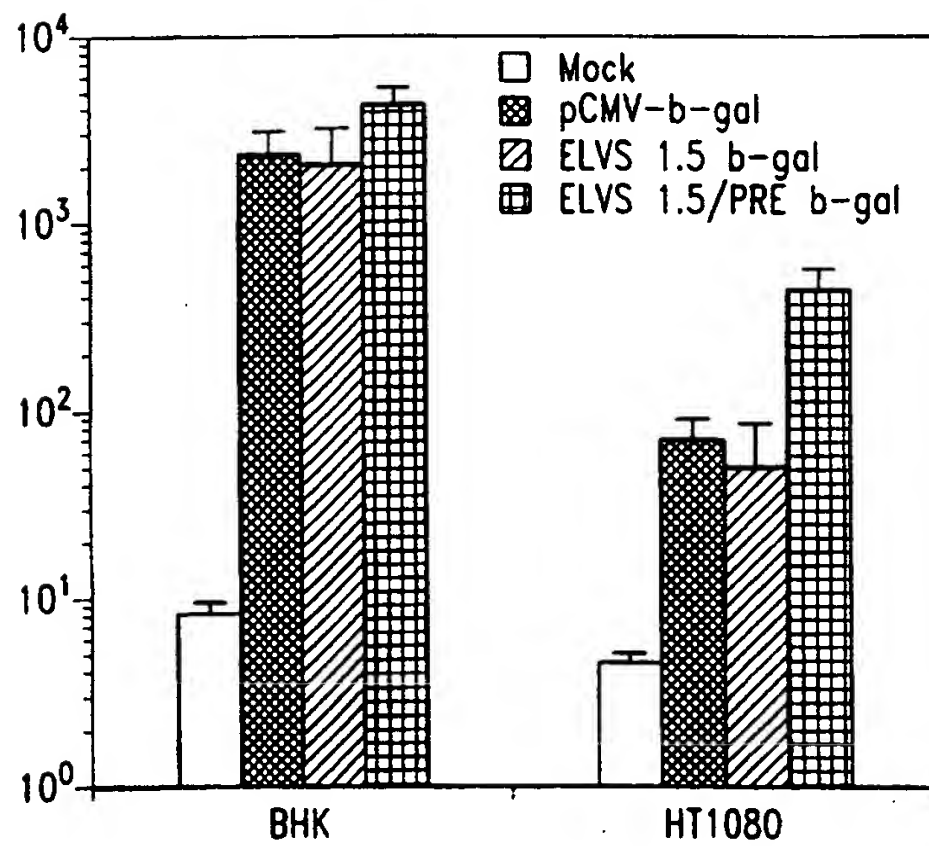


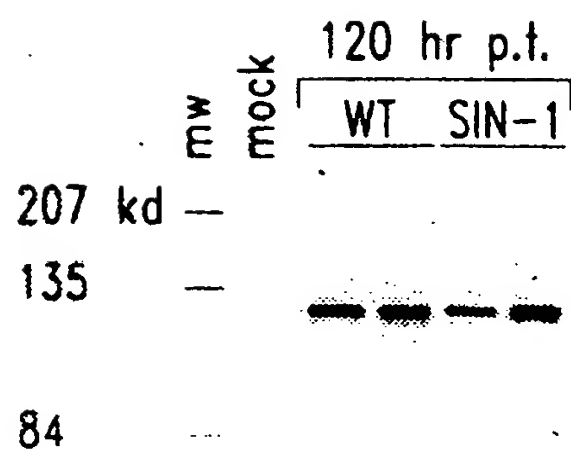
Fig. 13



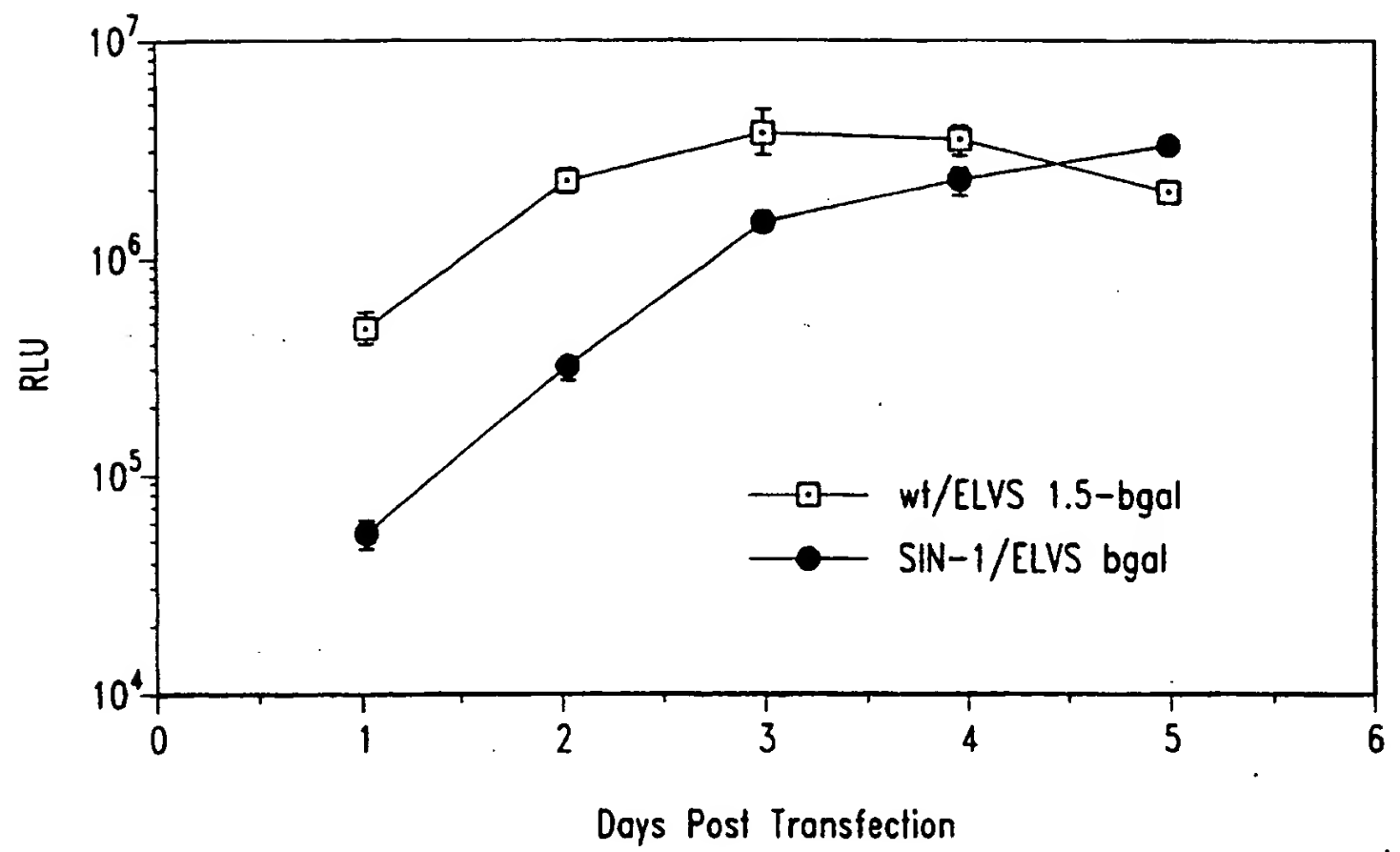
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*Fig. 12A**Fig. 12B*

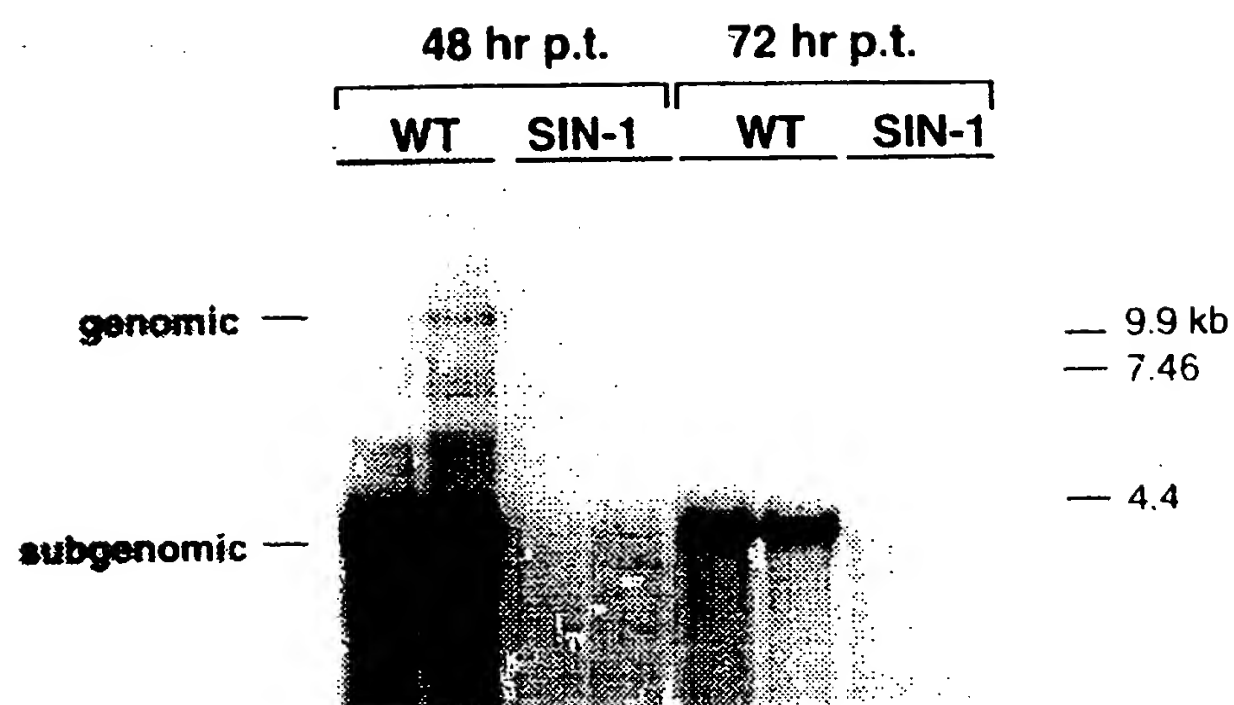
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*Fig. 11C*

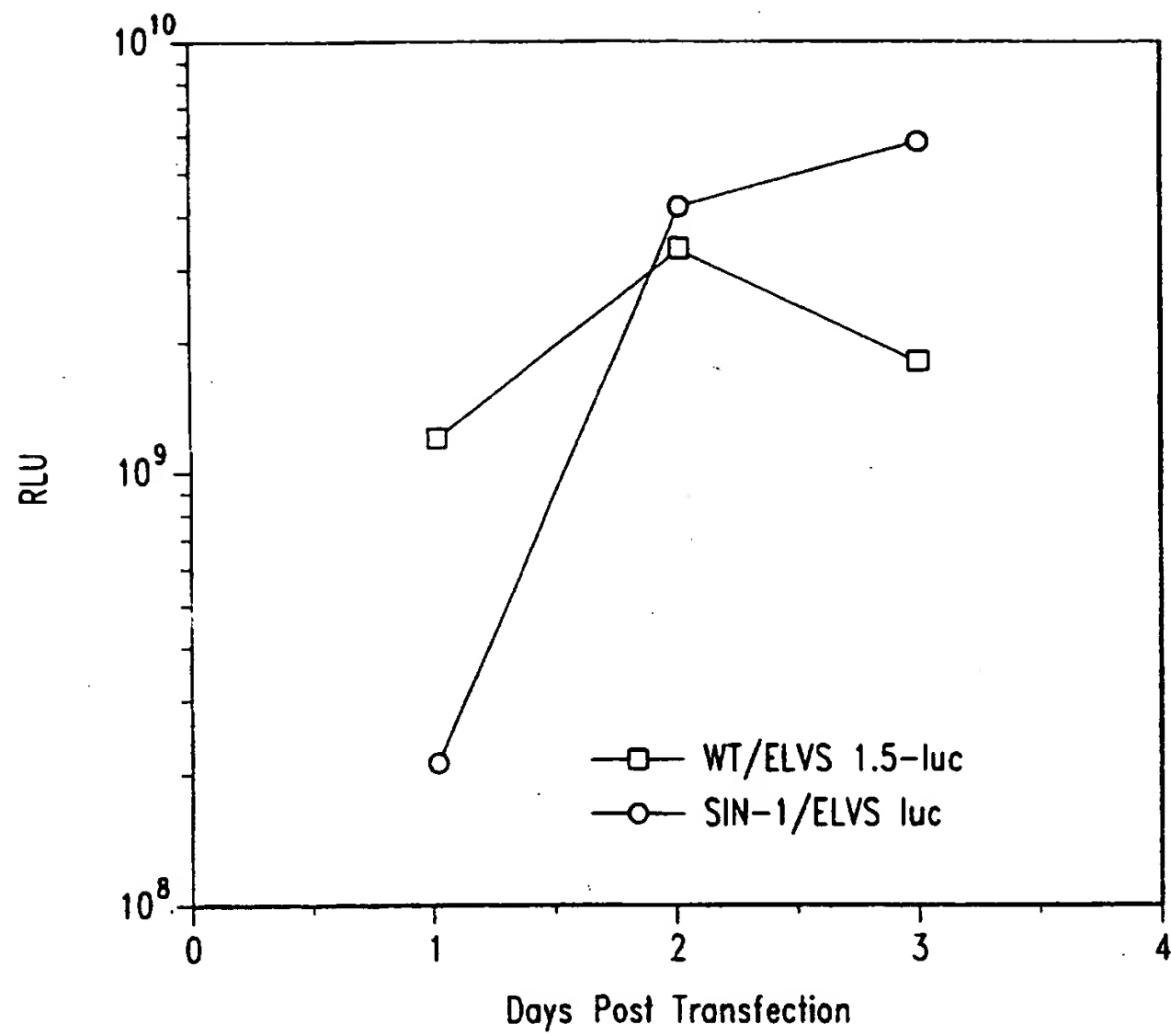
21/52 (A)

*Fig. 11B*

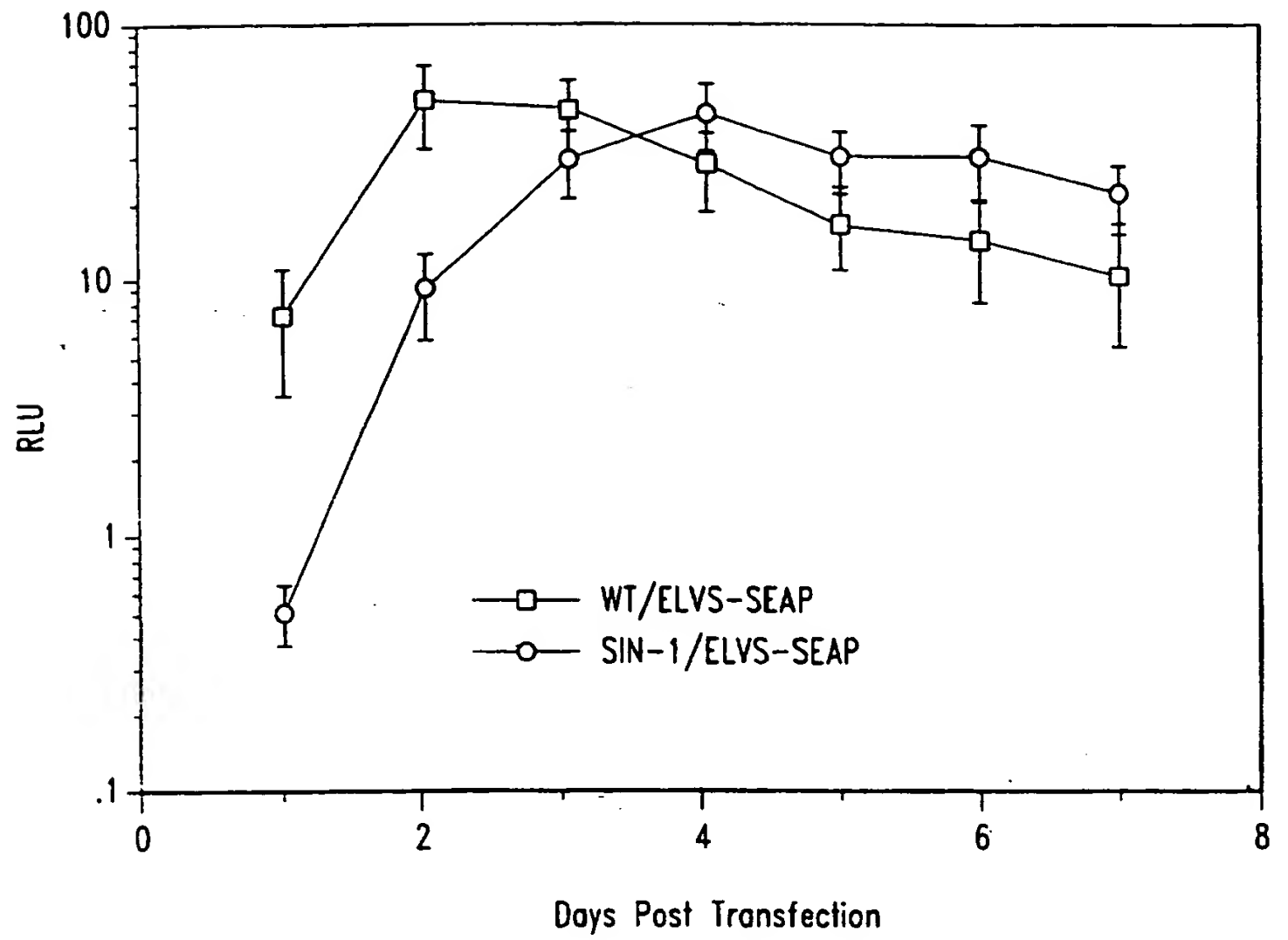
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*Fig. 11A*

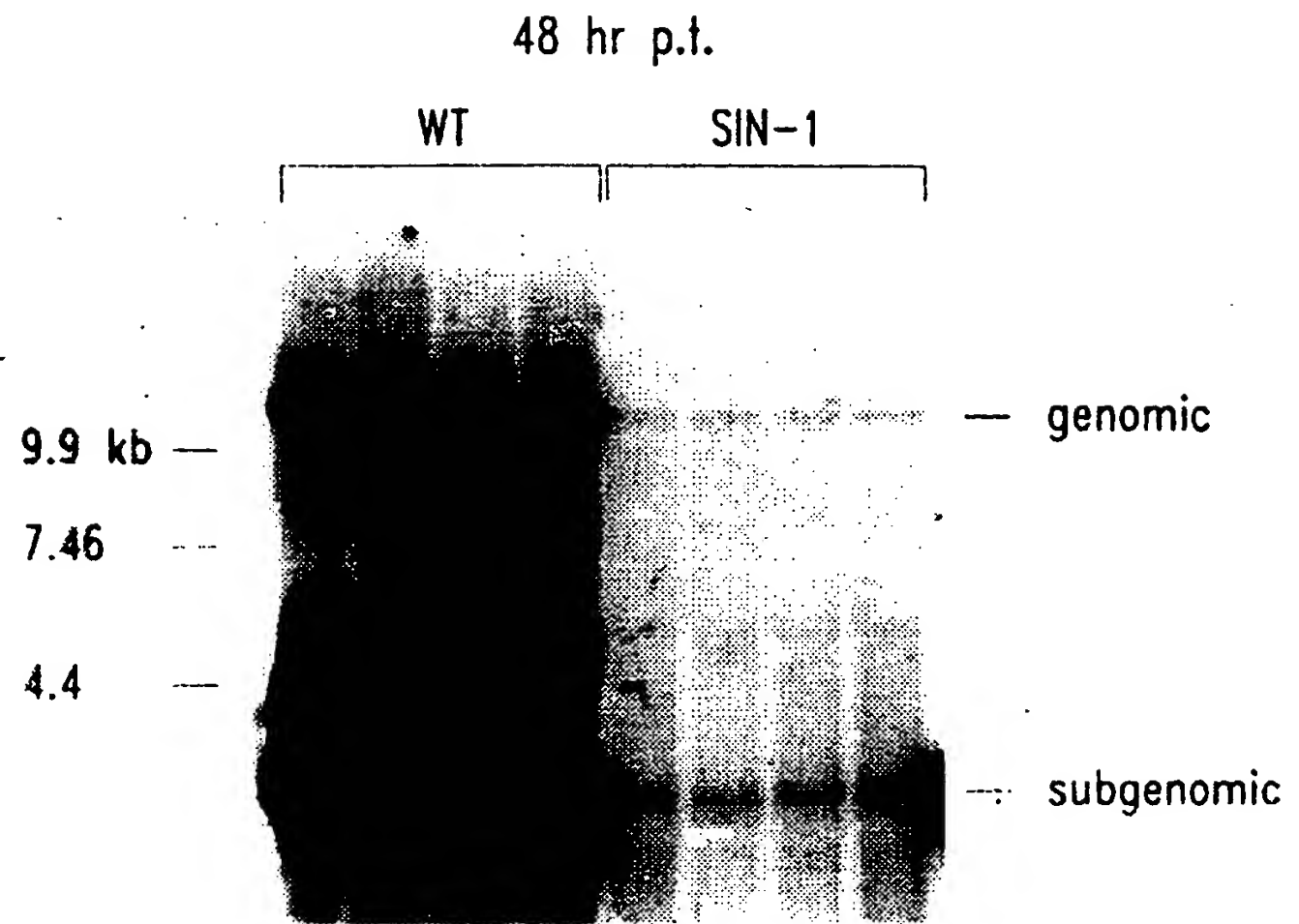
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*Fig. 10*

19/52 (A)

*Fig. 9B*

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*Fig. 9A*

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CTGTTCCAAA	TGTGCCACAG	ATACCGTATA	AGGCACTTGT	TGAAAGGGCA	GGGTATGCCC	10140
CGCTCAATTT	GGAGATCACT	GTCATGTCCT	CGGAGGTTTT	GCCTTCCACC	AACCAAGAGT	10200
ACATTACCTG	CAAATTCACC	ACTGTGGTCC	CCTCCCCAAA	AATCAAATGC	TGCGGCTCCT	10260
TGGAATGTCA	GCCGGCCGCT	CATGCAGACT	ATACCTGCAA	GGTCTTCGGA	GGGGTCTACC	10320
CCTTTATGTG	GGGAGGAGCG	CAATGTTTTT	GCGACAGTGA	GAACAGCCAG	ATGAGTGAGG	10380
CGTACGTCGA	ATTGTCAGCA	GATTGCGCGT	CTGACCACGC	GCAGGCGATT	AAGGTGCACA	10440
CTGCCGCGAT	GAAAGTAGGA	CTGCGTATTG	TGTACGGGAA	CACTACCAGT	TTCCTAGATG	10500
TGTACGTGAA	CGGAGTCACA	CCAGGAACGT	CTAAAGACTT	GAAAGTCATA	GCTGGACCAA	10560
TTTCAGCATC	GTTTACGCCA	TTCGATCATA	AGGTCGTTAT	CCATCGCGGC	CTGGTGTACA	10620
ACTATGACTT	CCCGGAATAT	GGAGCGATGA	AACCAGGAGC	GTTTGGAGAC	ATTCAAGCTA	10680
CCTCCTTGAC	TAGCAAGGAT	CTCATCGCCA	GCACAGACAT	TAGGCTACTC	AAGCCTTCCG	10740
CCAAGAACGT	GCATGTCCCG	TACACGCAGG	CCTCATCAGG	ATTTGAGATG	TGGAAAAACA	10800
ACTCAGGCCG	CCCACTGCAG	GAAACCGCAC	CTTTCGGGTG	TAAGATTGCA	GTAAATCCGC	10860
TCCGAGCGGT	GGACTGTTCA	TACGGGAACA	TTCCCATTTT	TATTGACATC	CCGAACGCTG	10920
CCTTTATCAG	GACATCAGAT	GCACCACTGG	TCTCAACAGT	CAAATGTGAA	GTCAGTGAGT	10980
GCACTTATTC	AGCAGACTTC	GGCGGGATGG	CCACCCTGCA	GTATGTATCC	GACCGCGAAG	11040
GTCAATGCCC	CGTACATTCC	CATTTCGAGCA	CAGCAACTCT	CCAAGAGTCG	ACAGTACATG	11100
TCCTGGAGAA	AGGAGCGGTG	ACAGTACACT	TAGCACCCGC	GAGTCCACAG	GCGAACTTTA	11160
TCGTATCGCT	GTGTGGGAAG	AAGACAACAT	GCAATGCAGA	ATGTAAACCA	CCAGCTGACC	11220
ATATCGTGAG	CACCCCGCAC	AAAAATGACC	AAGAATTTCA	AGCCGCCATC	TCAAAAACAT	11280
CATGGAGTTG	GCTGTTTGCC	CTTTTCGGCG	GCGCCTCGTC	GCTATTAATT	ATAGGACTTA	11340
TGATTTTTGC	TTGCAGCATG	ATGCTGACTA	GCACACGAAG	ATGACCGCTA	CGCCCCAATG	11400
ATCCGACCAG	CAAAACTCGA	TGTACTTCCG	AGGAACTGAT	GTGCATAATG	CATCAGGCTG	11460
GTACATTAGA	TCCCCGCTTA	CCGCGGGCAA	TATAGCAACA	CTAAAACTC	GATGTACTTC	11520
CGAGGAAGCG	CAGTGCATAA	TGCTGCGCAG	TGTTGCCACA	TAACCACTAT	ATTAACCATT	11580
TATCTAGCGG	ACGCCAAAAA	CTCAATGTAT	TTCTGAGGAA	GCGTGGTGCA	TAATGCCACG	11640
CAGCGTCTGC	ATAACTTTTA	TTATTTCTTT	TATTAATCAA	CAAAATTTTG	TTTTTAACAT	11700
TTCAAAAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA			11740

*Fig. 8E*

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TCAGAGGGGA	AATAAAGCAT	CTCTACGGTG	GTCCTAAATA	GTCAGCATAG	TACATTTTCAT	7620
CTGACTAATA	CTACAACACC	ACCACCATGA	ATAGAGGATT	CTTTAACATG	CTCGGCCGCC	7680
GCCCCCTCCC	GGCCCCCACT	GCCATGTGGA	GGCCGCGGAG	AAGGAGGCAG	GCGGCCCCGA	7740
TGCCTGCCCCG	CAACGGGCTG	GCTTCTCAA	TCCAGCAACT	GACCACAGCC	GTCAGTGCCC	7800
TAGTCATTGG	ACAGGCAACT	AGACCTCAAC	CCCCACGTCC	ACGCCCCGCA	CCGCGCCAGA	7860
AGAAGCAGGC	GCCCAAGCAA	CCACCGAAGC	CGAAGAAACC	AAAAACGCAG	GAGAAGAAGA	7920
AGAAGCAACC	TGCAAAACCC	AAACCCGGAA	AGAGACAGCG	CATGGCACTT	AAGTTGGAGG	7980
CCGACAGATT	GTTCGACGTC	AAGAACGAGG	ACGGAGATGT	CATCGGGCAC	GCACTGGCCA	8040
TGGAAGGAAA	GGTAATGAAA	CCTCTGCACG	TGAAAGGAAC	CATCGACCAC	CCTGTGCTAT	8100
CAAAGCTCAA	ATTTACCAAG	TCGTCAGCAT	ACGACATGGA	GTTCGCACAG	TTGCCAGTCA	8160
ACATGAGAAG	TGAGGCATTG	ACCTACACCA	GTGAACACCC	CGAAGGATTG	TATAACTGGC	8220
ACCACGGAGC	GGTGCAGTAT	AGTGGAGGTA	GATTTACCAT	CCCTCGCGGA	GTAGGAGGCA	8280
GAGGAGACAG	CGGTGCTCCG	ATCATGGATA	ACTCCGGTCG	GGTTGTCCCG	ATAGTCCTCG	8340
GTGGCGCTGA	TGAAGGAACA	CGAACTGCCC	TTTCGGTTCG	CACCTGGAAT	AGTAAAGGGA	8400
AGACAATTAA	GACGACCCCG	GAAGGGACAG	AAGAGTGGTC	CGCAGCACCA	CTGGTCACGG	8460
CAATGTGTTT	GCTCGGAAAT	GTGAGCTTCC	CATGCGACCG	CCCGCCCA	TGCTATACCC	8520
GCGAACCTTC	CAGAGCCCTC	GACATCCTTG	AAGAGAACGT	GAACCATGAG	GCCTACGATA	8580
CCCTGCTCAA	TGCCATATTG	CGGTGCGGAT	CGTCTGGCAG	AAGCAAAAGA	AGCGTCATTG	8640
ACGACTTTAC	CCTGACCAGC	CCCTACTTGG	GCACATGCTC	GTACTGCCAC	CATACTGTAC	8700
CGTGCTTCAG	CCCTGTTAAG	ATCGAGCAGG	TCTGGGACGA	AGCGGACGAT	AACACCATAC	8760
GCATACAGAC	TTCCGCCCAG	TTTGATACCG	ACCAAAGCGG	AGCAGCAAGC	GCAAACAAGT	8820
ACCGCTACAT	GTCGCTTAAG	CAGGATCACA	CCGTAAAGA	AGGCACCATG	GATGACATCA	8880
AGATTAGCAC	CTCAGGACCG	TGTAGAAGGC	TTAGCTACAA	AGGATACTTT	CTCCTCGCAA	8940
AATGCCCTCC	AGGGGACAGC	GTAACGGTTA	GCATAGTGAG	TAGCAACTCA	GCAACGTCAT	9000
GTACACTGGC	CCGCAAGATA	AAACCAAAAT	TCGTGGGACG	GGAAAAATAT	GATCTACCTC	9060
CCGTTACCGG	TAAAAAATT	CCTTGACACG	TGTACGACCG	TCTGAAAGAA	ACAACTGCAG	9120
GCTACATCAC	TATGCACAGG	CCGAGACCGC	ACGCTTATAC	ATCCTACCTG	GAAGAATCAT	9180
CAGGGAAAGT	TTACGCAAAG	CCGCCATCTG	GGAAGAACAT	TACGTATGAG	TGCAAGTGCG	9240
GCGACTACAA	GACCGGAACC	GTTTCGACCC	GCACCGAAAT	CACTGGTTGC	ACCGCCATCA	9300
AGCAGTGCGT	CGCCTATAAG	AGCGACCAAA	CGAAGTGGGT	CTTCAACTCA	CCGGACTTGA	9360
TCAGACATGA	CGACCACACG	GCCCAAGGGA	AATTGCATTT	GCCTTTCAAG	TTGATCCCGA	9420
GTACCTGCAT	GGTCCCTGTT	GCCCACGCGC	CGAATGTAAT	ACATGGCTTT	AAACACATCA	9480
GCCTCCAATT	AGATACAGAC	CACTTGACAT	TGCTCACCAC	CAGGAGACTA	GGGGCAAACC	9540
CGGAACCAAC	CACTGAATGG	ATCGTCGGAA	AGACGGTCAG	AAACTTCACC	GTCGACCGAG	9600
ATGGCCTGGA	ATACATATGG	GGAAATCATG	AGCCAGTGAG	GGTCTATGCC	CAAGAGTCAG	9660
CACCAGGAGA	CCCTCACGGA	TGGCCACACG	AAATAGTACA	GCATTACTAC	CATCGCCATC	9720
CTGTGTACAC	CATCTTAGCC	GTCGCATCAG	CTACCGTGGC	GATGATGATT	GGCGTAACTG	9780
TTGCAGTGTT	ATGTGCCTGT	AAAGCGCGCC	GTGAGTGCCT	GACGCCATAC	GCCCTGGCCC	9840
CAAACGCCGT	AATCCCAACT	TCGCTGGCAC	TCTTGTGCTG	CGTTAGGTGC	GCCAATGCTG	9900
AAACGTTTAC	CGAGACCATG	AGTTACTTGT	GGTCGAACAG	TCAGCCGTTT	TTCTGGGTCC	9960
AGTTGTGCAT	ACCTTTGGCC	GCTTTCATCG	TTCTAATGCG	CTGCTGCTCC	TGCTGCCTGC	10020
CTTTTTTAGT	GGTTGCCGGC	GCCTACCTGG	CGAAGGTAGA	CGCCTACGAA	CATGCGACCA	10080

*Fig. 8D*

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CGCACACTCC	CGCATTCTGT	CCCGCCCCGT	AGTACATAGA	AGTGCCAGAA	CAGCCTACCG	5100
CTCCTCCTGC	ACAGGCCGAG	GAGGCCCCCG	AAGTTGTAGC	GACACCGTCA	CCATCTACAG	5160
CTGATAACAC	CTCGCTTGAT	GTCACAGACA	TCTCACTGGA	TATGGATGAC	AGTAGCGAAG	5220
GCTCACTTTT	TTCGAGCTTT	AGCGGATCGG	ACAACTCTAT	TACTAGTATG	GACAGTTGGT	5280
CGTCAGGACC	TAGTTCACTA	GAGATAGTAG	ACCGAAGGCA	GGTGGTGGTG	GCTGACGTTT	5340
ATGCCGTCCA	AGAGCCTGCC	CCTATTCCAC	CGCCAAGGCT	AAAGAAGATG	GCCCCGCTGG	5400
CAGCGGCAAG	AAAAGAGCCC	ACTCCACCGG	CAAGCAATAG	CTCTGAGTCC	CTCCACCTCT	5460
CTTTTGGTGG	GGTATCCATG	TCCCTCGGAT	CAATTTTTCGA	CGGAGAGACG	GCCCCGCCAGG	5520
CAGCGGTACA	ACCCCTGGCA	ACAGGCCCCA	CGGATGTGCC	TATGTCTTTC	GGATCGTTTT	5580
CCGACGGAGA	GATTGATGAG	CTGAGCCGCA	GAGTAACTGA	GTCCGAACCC	GTCCTGTTTT	5640
GATCATTTGA	ACCGGGCGAA	GTGAACTCAA	TTATATCGTC	CCGATCAGCC	GTATCTTTTC	5700
CACTACGCAA	GCAGAGACGT	AGACGCAGGA	GCAGGAGGAC	TGAATACTGA	CTAACCAGGG	5760
TAGGTGGGTA	CATATTTTCG	ACGGACACAG	GCCCTGGGCA	CTTGCAAAAG	AAGTCCGTTT	5820
TGCAGAACCA	GCTTACAGAA	CCGACCTTGG	AGCGCAATGT	CCTGGAAAGA	ATTATGCCC	5880
CGGTGCTCGA	CACGTGAAA	GAGGAACAAC	TCAAACCTAG	GTACCAGATG	ATGCCACCG	5940
AAGCCAACAA	AAGTAGGTAC	CAGTCTCGTA	AAGTAGAAAA	TCAGAAAGCC	ATAACCACTG	6000
AGCGACTACT	GTCAGGACTA	CGACTGTATA	ACTCTGCCAC	AGATCAGCCA	GAATGCTATA	6060
AGATCACCTA	TCCGAAACCA	TTGTACTCCA	GTAGCGTACC	GGCGAACTAC	TCCGATCCAC	6120
AGTTCGCTGT	AGCTGTCTGT	AACAACATATC	TGCATGAGAA	CTATCCGACA	GTAGCATCTT	6180
ATCAGATTAC	TGACGAGTAC	GATGCTTACT	TGGATATGGT	AGACGGGACA	GTCGCCTGCC	6240
TGGATACTGC	AACCTTCTGC	CCCGCTAAGC	TTAGAAGTTA	CCCGAAAAAA	CATGAGTATA	6300
GAGCCCCGAA	TATCCGCAGT	GCGGTTCCAT	CAGCGATGCA	GAACACGCTA	CAAAATGTGC	6360
TCATTGCCGC	AACTAAAAGA	AATTGCAACG	TCACGCAGAT	GCGTGAACCTG	CCAACACTGG	6420
ACTCAGCGAC	ATTCAATGTC	GAATGCTTTC	GAAATATGTC	ATGTAATGAC	GAGTATTGGG	6480
AGGAGTTCGC	TCGGAAGCCA	ATTAGGATTA	CCACTGAGTT	TGTCACCGCA	TATGTAGCTA	6540
GACTGAAAGG	CCCTAAGGCC	GCCGCACTAT	TTGCAAAAGAC	GTATAATTTG	GTCCCATTGC	6600
AAGAAGTGCC	TATGGATAGA	TTCGTCTATG	ACATGAAAAG	AGACGTGAAA	GTTACACCAG	6660
GCACGAAACA	CACAGAAGAA	AGACCGAAAG	TACAAGTGAT	ACAAGCCGCA	GAACCCCTGG	6720
CGACTGCTTA	CTTATGCCGG	ATTCACCGGG	AATTAGTGCG	TAGGCTTACG	GCCGTCTTGC	6780
TTCCAAACAT	TCACACGCTT	TTTGACATGT	CGGCGGAGGA	TTTTGATGCA	ATCATAGCAG	6840
AACACTTCAA	GCAAGGCGAC	CCGGTACTGG	AGACGGATAT	CGCATCATTC	GACAAAAGCC	6900
AAGACGACGC	TATGGCGTTA	ACCGGTCTGA	TGATCTTGGA	GGACCTGGGT	GTGGATCAAC	6960
CACTACTCGA	CTTGATCGAG	TGCGCCTTTG	GAGAAATATC	ATCCACCCAT	CTACCTACGG	7020
GTACTCGTTT	TAAATTCCGG	GCGATGATGA	AATCCGGAAT	GTTCCCTACA	CTTTTGTGCA	7080
ACACAGTTTT	GAATGTCTGT	ATCGCCAGCA	GAGTACTAGA	AGAGCGGCTT	AAAACGTCCA	7140
GATGTGCAGC	GTTCAATTGG	GACGACAACA	TCATACATGG	AGTAGTATCT	GACAAAGAAA	7200
TGGCTGAGAG	GTGCGCCACC	TGGCTCAACA	TGGAGGTAA	GATCATCGAC	GCAGTCATCG	7260
GTGAGAGACC	ACCTTACTTC	TGCGGCGGAT	TTATCTTGCA	AGATTCCGTT	ACTTCCACAG	7320
CGTGCCCGCT	GGCGGATCCC	CTGAAAAGGC	TGTTTAAGTT	GGGTAACCG	CTCCCAGCCG	7380
ACGACGAGCA	AGACGAAGAC	AGAAGACGCG	CTCTGCTAGA	TGAAACAAAG	GCGTGGTTTA	7440
GAGTAGGTAT	AACAGGCACT	TTAGCAGTGG	CCGTGACGAC	CCGGTATGAG	GTAGACAATA	7500
TTACACCTGT	CCTACTGGCA	TTGAGAACTT	TTGCCAGAG	CAAAAGAGCA	TTCCAAGCCA	7560

*Fig. 8C*

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CCATGCAATG	CGGATTCTTC	AACATGATGC	AACTAAAGGT	ACATTTCAAT	CACCCTGAAA	2580
AAGACATATG	CACCAAGACA	TTCTACAAGT	ATATCTCCCG	GCGTTGCACA	CAGCCAGTTA	2640
CAGCTATTGT	ATCGACACTG	CATTACGATG	GAAAGATGAA	AACCACGAAC	CCGTGCAAGA	2700
AGAACATTGA	AATCGATATT	ACAGGGGCCA	CAAAGCCGAA	GCCAGGGGAT	ATCATCCTGA	2760
CATGTTTCCG	CGGGTGGGTT	AAGCAATTGC	AAATCGACTA	TCCCGGACAT	GAAGTAATGA	2820
CAGCCGCGGC	CTCACAAGGG	CTAACCAGAA	AAGGAGTGTA	TGCCGTCCGG	CAAAAAGTCA	2880
ATGAAAACCC	ACTGTACGCG	ATCACATCAG	AGCATGTGAA	CGTGTTGCTC	ACCCGCACTG	2940
AGGACAGGCT	AGTGTGGAAA	ACCTTGCAAG	GCGACCCATG	GATTAAGCAG	CCCACTAACA	3000
TACCTAAAGG	AAACTTTCAG	GCTACTATAG	AGGACTGGGA	AGCTGAACAC	AAGGGAATAA	3060
TTGCTGCAAT	AAACAGCCCC	ACTCCCCGTG	CCAATCCGTT	CAGCTGCAAG	ACCAACGTTT	3120
GCTGGGCGAA	AGCATTGGAA	CCGATACTAG	CCACGGCCGG	TATCGTACTT	ACCGGTTGCC	3180
AGTGGAGCGA	ACTGTTCCCA	CAGTTTGCGG	ATGACAAACC	ACATTCGGCC	ATTTACGCCT	3240
TAGACGTAAT	TTGCATTAAG	TTTTTCGGCA	TGGACTTGAC	AAGCGGACTG	TTTTCTAAAC	3300
AGAGCATCCC	ACTAACGTAC	CATCCCGCCG	ATTCAGCGAG	GCCGGTAGCT	CATTGGGACA	3360
ACAGCCCAGG	AACCCGCAAG	TATGGGTACG	ATCACGCCAT	TGCCGCCGAA	CTCTCCCGTA	3420
GATTTCCGGT	GTTCCAGCTA	GCTGGGAAGG	GCACACAAC	TGATTTGCAG	ACGGGGAGAA	3480
CCAGAGTTAT	CTCTGCACAG	CATAACCTGG	TCCCGGTGAA	CCGCAATCTT	CCTCACGCCT	3540
TAGTCCCCGA	GTACAAGGAG	AAGCAACCCG	GCCCGGTCAA	AAAATTCTTG	AACCAGTTCA	3600
AACACCACTC	AGTACTTGTG	GTATCAGAGG	AAAAAATTGA	AGCTCCCCGT	AAGAGAATCG	3660
AATGGATCGC	CCCGATTGGC	ATAGCCGGTG	CAGATAAGAA	CTACAACCTG	GCTTTCCGGT	3720
TTCCGCCGCA	GGCACGGTAC	GACCTGGTGT	TCATCAACAT	TGGAACATAA	TACAGAAACC	3780
ACCACTTTCA	GCAGTGCGAA	GACCATGCGG	CGACCTTAAA	AACCCTTTTC	CGTTCCGGCC	3840
TGAATTGCCT	TAACCCAGGA	GGCACCCCTC	TGGTGAAGTC	CTATGGCTAC	GCCGACCGCA	3900
ACAGTGAGGA	CGTAGTCACC	GCTCTTGCCA	GAAAGTTTGT	CAGGGTGTCT	GCAGCGAGAC	3960
CAGATTGTGT	CTCAAGCAAT	ACAGAAATGT	ACCTGATTTT	CCGACAATA	GACAACAGCC	4020
GTACACGGCA	ATTCACCCCG	CACCATCTGA	ATTGCGTGAT	TTCGTCCGTG	TATGAGGGTA	4080
CAAGAGATGG	AGTTGGAGCC	GCGCCGTCAT	ACCGCACCAA	AAGGGAGAAT	ATTGCTGACT	4140
GTCAAGAGGA	AGCAGTTGTC	AACGCAGCCA	ATCCGCTGGG	TAGACCAGGC	GAAGGAGTCT	4200
GCCGTGCCAT	CTATAAACGT	TGGCCGACCA	GTTTTACCGA	TTCAGCCACG	GAGACAGGCA	4260
CCGCAAGAAT	GACTGTGTGC	CTAGGAAAGA	AAGTGATCCA	CGCGGTCGGC	CCTGATTTCC	4320
GGAAGCACCC	AGAAGCAGAA	GCCTTGAAAT	TGCTACAAAA	CGCCTACCAT	GCAGTGGCAG	4380
ACTTAGTAAA	TGAACATAAC	ATCAAGTCTG	TCGCCATTCC	ACTGCTATCT	ACAGGCATTT	4440
ACGCAGCCGG	AAAAGACCGC	CTTGAAGTAT	CACTTAACTG	CTTGACAACC	GCGCTAGACA	4500
GAAGTGACGC	GGACGTAACC	ATCTATTGCC	TGGATAAGAA	GTGGAAGGAA	AGAATCGACG	4560
CGGCACTCCA	ACTTAAGGAG	TCTGTAACAG	AGCTGAAGGA	TGAAGATATG	GAGATCGACG	4620
ATGAGTTAGT	ATGGATCCAT	CCAGACAGTT	GCTTGAAGGG	AAGAAAGGGA	TTCAGTACTA	4680
CAAAAGGAAA	ATTGTATTCC	TACTTCGAAG	GCACCAAAAT	CCATCAAGCA	GCAAAAGACA	4740
TGGCGGAGAT	AAAGGTCCTG	TTCCCTAATG	ACCAGGAAAG	TAATGAACAA	CTGTGTGCCT	4800
ACATATTGGG	TGAGACCATG	GAAGCAATCC	GCGAAAAGTG	CCCGGTCGAC	CATAACCCGT	4860
CGTCTAGCCC	GCCCAAAACG	TTGCCGTGCC	TTTGATGTGA	TGCCATGACG	CCAGAAAGGG	4920
TCCACAGACT	TAGAAGCAAT	AACGTCAAAG	AAGTTACAGT	ATGCTCCTCC	ACCCCCCTTC	4980
CTAAGCACAA	AATTAAGAAT	GTTCAGAAGG	TTCAGTGCAC	GAAAGTAGTC	CTGTTTAATC	5040

*Fig. 8B*

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ATTGACGGCG	TAGTACACAC	TATTGAATCA	AACAGCCGAC	CAATTGCACT	ACCATCACAA	60
TGGAGAAGCC	AGTAGTAAAC	GTAGACGTAG	ACCCCCAGAG	TCCGTTTGTC	GTGCAACTGC	120
AAAAAAGCTT	CCCGCAATTT	GAGGTAGTAG	CACAGCAGGT	CACTCCAAAT	GACCATGCTA	180
ATGCCAGAGC	ATTTTCGCAT	CTGGCCAGTA	AACTAATCGA	GCTGGAGGTT	CCTACCACAG	240
CGACGATCTT	GGACATAGGC	AGCGCACCGG	CTCGTAGAAT	GTTTTCCGAG	CACCAGTATC	300
ATTGTGTCTG	CCCCATGCGT	AGTCCAGAAG	ACCCGGACCG	CATGATGAAA	TACGCCAGTA	360
AACTGGCGGA	AAAAGCGTGC	AAGATTACAA	ACAAGAACTT	GCATGAGAAG	ATTAAGGATC	420
TCCGGACCGT	ACTTGATACG	CCGGATGCTG	AAACACCATC	GCTCTGCTTT	CACAACGATG	480
TTACCTGCAA	CATGCGTGCC	GAATATTCCG	TCATGCAGGA	CGTGTATATC	AACGCTCCCG	540
GAACATCTA	TCATCAGGCT	ATGAAAGGCG	TGCGGACCCT	GTA CTGGATT	GGCTTCGACA	600
CCACCCAGTT	CATGTTCTCG	GCTATGGCAG	GTTCTGTACC	TGCGTACAAC	ACCAACTGGG	660
CCGACGAGAA	AGTCCTTGAA	GCGCGTAACA	TCGGACTTTG	CAGCACAAAG	CTGAGTGAAG	720
GTAGGACAGG	AAAATTGTCT	ATAATGAGGA	AGAAGGAGTT	GAAGCCCGGG	TCGCGGGTTT	780
ATTTCTCCGT	AGGATCGACA	CTTTATCCAG	AACACAGAGC	CAGCTTGCA	AGCTGGCATC	840
TTCCATCGGT	GTTCCACTTG	AATGGAAAGC	AGTCGTACAC	TTGCCGCTGT	GATACAGTGG	900
TGAGTTGCGA	AGGCTACGTA	GTGAAGAAAA	TCACCATCAG	TCCCGGGATC	ACGGGAGAAA	960
CCGTGGGATA	CGCGGTTACA	CACAATAGCG	AGGGCTTCTT	GCTATGCAAA	GTTACTGACA	1020
CAGTAAAAGG	AGAACGGGTA	TCGTTCCCTG	TGTGCACGTA	CATCCCGGCC	ACCATATGCG	1080
ATCAGATGAC	TGGTATAATG	GCCACGGATA	TATCACCTGA	CGATGCACAA	AAACTTCTGG	1140
TTGGGCTCAA	CCAGCGAATT	GTCATTAACG	GTAGGACTAA	CAGGAACACC	AACACCATGC	1200
AAAATTACCT	TCTGCCGATC	ATAGCACAA	GGTTCAGCAA	ATGGGCTAAG	GAGCGCAAGG	1260
ATGATCTTGA	TAACGAGAAA	ATGCTGGGTA	CTAGAGAACG	CAAGCTTACG	TATGGCTGCT	1320
TGTGGGCGTT	TCGCACTAAG	AAAGTACATT	CGTTTTATCG	CCCACCTGGA	ACGCAGACCT	1380
GCGTAAAAGT	CCCAGCCTCT	TTTAGCGCTT	TTCCCATGTC	GTCCGTATGG	ACGACCTCTT	1440
TGCCCATGTC	GCTGAGGCAG	AAATTGAAAC	TGGCATTGCA	ACCAAAGAAG	GAGGAAAAAC	1500
TGCTGCAGGT	CTCGGAGGAA	TTAGTCATGG	AGGCCAAGGC	TGCTTTTGAG	GATGCTCAGG	1560
AGGAAGCCAG	AGCGGAGAAG	CTCCGAGAAG	CACTTCCACC	ATTAGTGGCA	GACAAAGGCA	1620
TCGAGGCAGC	CGCAGAAGTT	GTCTGCGAAG	TGGAGGGGCT	CCAGGCGGAC	ATCGGAGCAG	1680
CATTAGTTGA	AACCCCGCGC	GGTCACGTAA	GGATAATACC	TCAAGCAAAT	GACCGTATGA	1740
TCGGACAGTA	TATCGTTGTC	TCGCCAAACT	CTGTGCTGAA	GAATGCCAAA	CTCGCACCAG	1800
CGCACCCGCT	AGCAGATCAG	GTTAAGATCA	TAACACACTC	CGGAAGATCA	GGAAGGTACG	1860
CGGTGCAACC	ATACGACGCT	AAAGTACTGA	TGCCAGCAGG	AGGTGCCGTA	CCATGGCCAG	1920
AATTCCTAGC	ACTGAGTGAG	AGCGCCACGT	TAGTGTACAA	CGAAAGAGAG	TTTGTGAACC	1980
GCAAACATA	CCACATTGCC	ATGCATGGCC	CCGCCAAGAA	TACAGAAGAG	GAGCAGTACA	2040
AGGTTACAAA	GGCAGAGCTT	GCAGAAACAG	AGTACGTGTT	TGACGTGGAC	AAGAAGCGTT	2100
GCGTTAAGAA	GGAGAAGCC	TCAGGTCTGG	TCCTCTCGGG	AGAACTGACC	AACCCTCCCT	2160
ATCATGAGCT	AGCTCTGGAG	GGACTGAAGA	CCCGACCTGC	GGTCCCGTAC	AAGGTGCGAA	2220
CAATAGGAGT	GATAGGCACA	CCGGGGTCCG	GCAAGTCAGC	TATTATCAAG	TCAACTGTCA	2280
CGGCACGAGA	TCTTGTTACC	AGCGGAAAGA	AAGAAAATTG	TCGCGAAATT	GAGGCCGACG	2340
TGCTAAGACT	GAGGGGTATG	CAGATTACGT	CGAAGACAGT	AGATTCCGTT	ATGCTCAACG	2400
GATGCCACAA	AGCCGTAGAA	GTGCTGTACG	TTGACGAAGC	GTTCCGCTGC	CACGCAGGAG	2460
CACTACTTGC	CTTGATTGCT	ATCGTCAGGC	CCCGCAAGAA	GGTAGTACTA	TGCGGAGACC	2520

*Fig. 8A*

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TCAGAGGGGA	AATAAAGCAT	CTCTACGGTG	GTCCTAAATA	GTCAGCATAG	TACATTTTCA	7620
CTGACTAATA	CTACAACACC	ACCACCATGA	ATAGAGGATT	CTTTAACATG	CTCGGCCGCC	7680
GCCCCTTCCC	GGCCCCCACT	GCCATGTGGA	GGCCGCCGAG	AAGGAGGCAG	GCGGCCCCGA	7740
TGCCTGCCCC	CAACGGGCTG	GCTTCTCAA	TCCAGCAACT	GACCACAGCC	GTCAGTGCCC	7800
TAGTCATTGG	ACAGGCAACT	AGACCTCAAC	CCCCACGTCC	ACGCCCCGCC	CCGCGCCAGA	7860
AGAAGCAGGC	GCCCAAGCAA	CCACCGAAGC	CGAAGAAACC	AAAAACGCAG	GAGAAGAAGA	7920
AGAAGCAACC	TGCAAAACCC	AAACCCGGAA	AGAGACAGCG	CATGGCACTT	AAGTTGGAGG	7980
CCGACAGATT	GTTCCGACGC					8000

*Fig. 7D*

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CGCACACTCC	CGCATTGCTT	CCCGCCCGTA	AGTACATAGA	AGTGCCAGAA	CAGCCTACCG	5100
CTCCTCCTGC	ACAGGCCGAG	GAGGCCCCCG	AAGTTGTAGC	GACACCGTCA	CCATCTACAG	5160
CTGATAACAC	CTCGCTTGAT	GTCACAGACA	TCTCACTGGA	TATGGATGAC	AGTAGCGAAG	5220
GCTCACTTTT	TTCGAGCTTT	AGCGGATCGG	ACAACCTCTAT	TACTAGTATG	GACAGTTGGT	5280
CGTCAGGACC	TAGTTCACTA	GAGATAGTAG	ACCGAAGGCA	GGTGGTGGTG	GCTGACGTTT	5340
ATGCCGTCCA	AGAGCCTGCC	CCTATTCCAC	CGCCAAGGCT	AAAGAAGATG	GCCCGCCTGG	5400
CAGCGGCAAG	AAAAGAGCCC	ACTCCACCGG	CAAGCAATAG	CTCTGAGTCC	CTCCACCTCT	5460
CTTTTGGTGG	GGTATCCATG	TCCCTCGGAT	CAATTTTCTGA	CGGAGAGACG	GCCCGCCAGG	5520
CAGCGGTACA	ACCCCTGGCA	ACAGGCCCCA	CGGATGTGCC	TATGTCTTTC	GGATCGTTTT	5580
CCGACGGAGA	GATTGATGAG	CTGAGCCGCA	GAGTAACTGA	GTCCGAACCC	GTCCTGTTTG	5640
GATCATTTGA	ACCGGGCGAA	GTGAACTCAA	TTATATCGTC	CCGATCAGCC	GTATCTTTTC	5700
CACTACGCAA	GCAGAGACGT	AGACGCAGGA	GCAGGAGGAC	TGAATACTGA	CTAACCGGGG	5760
TAGGTGGGTA	CATATTTTCTG	ACGGACACAG	GCCCTGGGCA	CTTGCAAAAG	AAGTCCGTTT	5820
TGCAGAACCA	GCTTACAGAA	CCGACCTTGG	AGCGCAATGT	CCTGGAAAGA	ATTCATGCCC	5880
CGGTGCTCGA	CACGTGCGAA	GAGGAACAAC	TCAAACCTCAG	GTACCAGATG	ATGCCACCCG	5940
AAGCCAACAA	AAGTAGGTAC	CAGTCTCGTA	AAGTAGAAAA	TCAGAAAGCC	ATAACCACTG	6000
AGCGACTACT	GTCAGGACTA	CGACTGTATA	ACTCTGCCAC	AGATCAGCCA	GAATGCTATA	6060
AGATCACCTA	TCCGAAACCA	TTGTACTCCA	GTAGCGTACC	GGCGAACTAC	TCCGATCCAC	6120
AGTTCGCTGT	AGCTGTCTGT	AACAACCTATC	TGCATGAGAA	CTATCCGACA	GTAGCATCTT	6180
ATCAGATTAC	TGACGAGTAC	GATGCTTACT	TGGATATGGT	AGACGGGACA	GTCGCCTGCC	6240
TGGATACTGC	AACCTTCTGC	CCCGCTAAGC	TTAGAAGTTA	CCCGAAAAAA	CATGAGTATA	6300
GAGCCCCGAA	TATCCGCAGT	GCGGTTCAT	CAGCGATGCA	GAACACGCTA	CAAAATGTGC	6360
TCATTGCCGC	AACTAAAAGA	AATTGCAACG	TCACGCAGAT	GCGTGAACTG	CCAACACTGG	6420
ACTCAGCGAC	ATTCAATGTC	GAATGCTTTC	GAAATATGTC	ATGTAATGAC	GAGTATTGGG	6480
AGGAGTTCGC	TCGGAAGCCA	ATTAGGATTA	CCACTGAGTT	TGTCACCGCA	TATGTAGCTA	6540
GACTGAAAGG	CCCTAAGGCC	GCCGCACTAT	TTGCAAAGAC	GTATAATTTG	GTCCCATTGC	6600
AAGAAGTGCC	TATGGATAGA	TTGTCATGG	ACATGAAAAG	AGACGTGAAA	GTTACACCAG	6660
GCACGAAACA	CACAGAAGAA	AGACCGAAAG	TACAAGTGAT	ACAAGCCGCA	GAACCCCTGG	6720
CGACTGCTTA	CTTATGCGGG	ATTCACCGGG	AATTAGTGCG	TAGGCTTACG	GCCGTCTTGC	6780
TTCCAAACAT	TCACACGCTT	TTTGACATGT	CGGCGGAGGA	TTTTGATGCA	ATCATAGCAG	6840
AACACTTCAA	GCAAGGCGAC	CCGGTACTGG	AGACGGATAT	CGCATCATTC	GACAAAAGCC	6900
AAGACGACGC	TATGGCGTTA	ACCGGTCTGA	TGATCTTGGA	GGACCTGGGT	GTGGATCAAC	6960
CACTACTCGA	CTTGATCGAG	TGCGCCTTTG	GAGAAATATC	ATCCACCCAT	CTACCTACGG	7020
GTAICTGTTT	TAAATTGCGG	GCGATGATGA	AATCCGGAAT	GTTCTCACA	CTTTTGTCA	7080
ACACAGTTTT	GAATGTCGTT	ATCGCCAGCA	GAGTACTAGA	AGAGCGGCTT	AAAACGTCCA	7140
GATGTGCAGC	GTTTATTGGC	GACGACAACA	TCATACATGG	AGTAGTATCT	GACAAAGAAA	7200
TGGCTGAGAG	GTGCGCCACC	TGGCTCAACA	TGGAGGTAA	GATCATCGAC	GCAGTCATCG	7260
GTGAGAGACC	ACCTTACTTC	TGCGGCGGAT	TTATCTTGCA	AGATTGCGTT	ACTTCCACAG	7320
CGTGCCGCGT	GGCGGATCCC	CTGAAAAGGC	TGTTTAAGTT	GGGTAAACCG	CTCCAGCCG	7380
ACGACGAGCA	AGACGAAGAC	AGAAGACGCG	CTCTGCTAGA	TGAAACAAAG	GCGTGGTTTA	7440
GAGTAGGTAT	AACAGGCACT	TTAGCAGTGG	CCGTGACGAC	CCGGTATGAG	GTAGACAATA	7500
TTACACCTGT	CCTACTGGCA	TTGAGAACTT	TTGCCAGAG	CAAAAGAGCA	TTCCAAGCCA	7560

*Fig. 7C*

SUBSTITUTE SHEET (RULE 26)



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CCATGCAATG	CGGATTCTTC	AACATGATGC	AACTAAAGGT	ACATTTCAAT	CACCCTGAAA	2580
AAGACATATG	CACCAAGACA	TTCTACAAGT	ATATCTCCCG	GCGTTGCACA	CAGCCAGTTA	2640
CAGCTATTGT	ATCGACACTG	CATTACGATG	GAAAGATGAA	AACCACGAAC	CCGTGCAAGA	2700
AGAACATTGA	AATCGATATT	ACAGGGGCCA	CAAAGCCGAA	GCCAGGGGAT	ATCATCCTGA	2760
CATGTTTCCG	CGGGTGGGTT	AAGCAATTGC	AAATCGACTA	TCCCGGACAT	GAAGTAATGA	2820
CAGCCGCGGC	CTCACAAGGG	CTAACCAGAA	AAGGAGTGTA	TGCCGTCCGG	CAAAAAGTCA	2880
ATGAAAACCC	ACTGTACCGG	ATCACATCAG	AGCATGTGAA	CGTGTGCTC	ACCCGCACTG	2940
AGGACAGGCT	AGTGTGGAAA	ACCTTGCAGG	GCGACCCATG	GATTAAGCAG	CCCACTAACA	3000
TACCTAAAGG	AAACTTTTCA	GCTACTATAG	AGGACTGGGA	AGCTGAACAC	AAGGGAATAA	3060
TTGCTGCAAT	AAACAGCCCC	ACTCCCCGTG	CCAATCCGTT	CAGCTGCAAG	ACCAACGTTT	3120
GCTGGGCGAA	AGCATTGGAA	CCGATACTAG	CCACGGCCGG	TATCGTACTT	ACCGGTTGCC	3180
AGTGGAGCGA	ACTGTTCCCA	CAGTTTGGCG	ATGACAAACC	ACATTCCGGC	ATTTACGCCT	3240
TAGACGTAAT	TTGCATTAAG	TTTTTCGGCA	TGGACTTGAC	AAGCGGACTG	TTTTCTAAAC	3300
AGAGCATCCC	ACTAACGTAC	CATCCCGCCG	ATTAGCGGAG	GCCGGTAGCT	CATTGGGACA	3360
ACAGCCCAGG	AACCCGCAAG	TATGGGTACG	ATCAGCCCAT	TGCCGCCGAA	CTCTCCCGTA	3420
GATTTCCGGT	GTTCCAGCTA	GCTGGGAAGG	GCACACAAC	TGATTTGCAG	ACGGGGAGAA	3480
CCAGAGTTAT	CTCTGCACAG	CATAACCTGG	TCCCGGTGAA	CCGCAATCTT	CCTCACGCCT	3540
TAGTCCCCGA	GTACAAGGAG	AAGCAACCCG	GCCCGGTCAA	AAAATTCTTG	AACCAGTTCA	3600
AACACCACTC	AGTACTTGTC	GTATCAGAGG	AAAAAATTGA	AGCTCCCCGT	AAGAGAATCG	3660
AATGGATCGC	CCCGATTGGC	ATAGCCGGTG	CAGATAAGAA	CTACAACCTG	GCTTTCGGGT	3720
TTCCGCCGCA	GGCACGGTAC	GACCTGGTGT	TCATCAACAT	TGGAACATAA	TACAGAAACC	3780
ACCACTTTCA	GCAGTGCGAA	GACCATGCGG	CGACCTTAAA	AACCTTTTCG	CGTTCGGCCC	3840
TGAATTGCCT	TAACCCAGGA	GGCACCTCG	TGGTGAAGTC	CTATGGCTAC	GCCGACCGCA	3900
ACAGTGAGGA	CGTAGTCACC	GCTCTTGCCA	GAAAGTTTGT	CAGGGTGTCT	GCAGCGAGAC	3960
CAGATTGTGT	CTCAAGCAAT	ACAGAAATGT	ACCTGATTTT	CCGACAACCTA	GACAACAGCC	4020
GTACACGGCA	ATTCACCCCG	CACCATCTGA	ATTGCGTGAT	TTCGTCCGTG	TATGAGGGTA	4080
CAAGAGATGG	AGTTGGAGCC	GCGCCGTCAT	ACCGCACCAA	AAGGGAGAAT	ATTGCTGACT	4140
GTCAAGAGGA	AGCAGTTGTC	AACGCAGCCA	ATCCGCTGGG	TAGACCAGGC	GAAGGAGTCT	4200
GCCGTGCCAT	CTATAAACGT	TGGCCGACCA	GTTTTACCGA	TTCAGCCACG	GAGACAGGCA	4260
CCGCAAGAAT	GACTGTGTGC	CTAGGAAAGA	AAGTGATCCA	CGCGGTCCGG	CCTGATTTCC	4320
GGAAGCACCC	AGAAGCAGAA	GCCTTGAAAT	TGCTACAAAA	CGCCTACCAT	GCAGTGGCAG	4380
ACTTAGTAAA	TGAACATAAC	ATCAAGTCTG	TCGCCATTCC	ACTGCTATCT	ACAGGCATTT	4440
ACGCAGCCGG	AAAAGACCGC	CTTGAAGTAT	CACCTAACTG	CTTGACAACC	GCGCTAGACA	4500
GAACTGACGC	GGACGTAAAC	ATCTATTGCC	TGGATAAGAA	GTGGAAGGAA	AGAATCGACG	4560
CGGCACTCCA	ACTTAAGGAG	TCTGTAACAG	AGCTGAAGGA	TGAAGATATG	GAGATCGACG	4620
ATGAGTTAGT	ATGGATCCAT	CCAGACAGTT	GCTTGAAGGG	AAGAAAGGGA	TTCAGTACTA	4680
CAAAAGGAAA	ATTGTATTCT	TACTTCGAAG	GCACCAAATT	CCATCAAGCA	GCAAAAGACA	4740
TGGCGGAGAT	AAAGGTCCTG	TTCCCTAATG	ACCAGGAAAG	TAATGAACAA	CTGTGTGCCT	4800
ACATATTGGG	TGAGACCATG	GAAGCAATCC	GCGAAAAGTG	CCCGGTCCGAC	CATAACCCGT	4860
CGTCTAGCCC	GCCCAAAACG	TTGCCGTGCC	TTTGCAATGA	TGCCATGACG	CCAGAAAGGG	4920
TCCACAGACT	TAGAAGCAAT	AACGTCAAAG	AAGTTACAGT	ATGCTCCTCC	ACCCCCCTTC	4980
CTAAGCACAA	AATTAAGAAT	GTTCAGAAGG	TTCAGTGCAC	GAAAGTAGTC	CTGTTTAATC	5040

*Fig. 7B*

SUBSTITUTE SHEET (RULE 26)

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ATTGACGGCG	TAGTACACAC	TATTGAATCA	AACAGCCGAC	CAATTGCACT	ACCATCACAA	60
TGGAGAAGCC	AGTAGTAAAC	GTAGACGTAG	ACCCCAGAG	TCCGTTTGTC	GTGCAACTGC	120
AAAAAAGCTT	CCCGCAATT	GAGGTAGTAG	CACAGCAGGT	CACTCCAAAT	GACCATGCTA	180
ATGCCAGAGC	ATTTTCGCAT	CTGGCCAGTA	AACTAATCGA	GCTGGAGGT	CCTACCACAG	240
CGACGATCTT	GGACATAGGC	AGCGCACCGG	CTCGTAGAAT	GTTTTCCGAG	CACCAGTATC	300
ATTGTGTCTG	CCCCATGCGT	AGTCCAGAAG	ACCCGGACCG	CATGATGAAA	TACGCCAGTA	360
AACTGGCGGA	AAAAGCGTGC	AAGATTACAA	ACAAGAACTT	GCATGAGAAG	ATTAAGGATC	420
TCCGGACCGT	ACTTGATACG	CCGGATGCTG	AAACACCATC	GCTCTGCTTT	CACAACGATG	480
TTACCTGCAA	CATGCGTGCC	GAATATTCCG	TCATGCAGGA	CGTGTATATC	AACGCTCCCC	540
GAACATCTA	TCATCAGGCT	ATGAAAGGCG	TGCGGACCC	GTACTGGATT	GGCTTCGACA	600
CCACCCAGTT	CATGTTCTCG	GCTATGGCAG	GTTCTGACCC	TGCGTACAAC	ACCAACTGGG	660
CCGACGAGAA	AGTCCTTGAA	GCGCGTAACA	TCGGACTTTG	CAGCACAAAG	CTGAGTGAAG	720
GTAGGACAGG	AAAATTGTCTG	ATAATGAGGA	AGAAGGAGTT	GAAGCCCGGG	TCGCGGGTTT	780
ATTTCTCCGT	AGGATCGACA	CTTTATCCAG	AACACAGAGC	CAGCTTGCA	AGCTGGCATC	840
TTCCATCCGT	GTTCCACTTG	AATGGAAAGC	AGTCGTACAC	TTGCCGCTGT	GATACAGTGG	900
TGAGTTGCGA	AGGCTACGTA	GTGAAGAAAA	TCACCATCAG	TCCCGGGATC	ACGGGAGAAA	960
CCGTGGGATA	CGCGGTTACA	CACAATAGCG	AGGGCTTCTT	GCTATGCAAA	GTTACTGACA	1020
CAGTAAAAGG	AGAACGGGTA	TCGTTCCCTG	TGTGCACGTA	CATCCCGGCC	ACCATATGCC	1080
ATCAGATGAC	TGGTATAATG	GCCACGGATA	TATCACCTGA	CGATGCACAA	AAACTTCTGG	1140
TTGGGCTCAA	CCAGCGAATT	GTCATTAACG	GTAGGACTAA	CAGGAACACC	AACACCATGC	1200
AAAATTACCT	TCTGCCGATC	ATAGCACAA	GGTTCAGCAA	ATGGGCTAAG	GAGCGCAAGG	1260
ATGATCTTGA	TAACGAGAAA	ATGCTGGGTA	CTAGAGAACC	CAAGCTTACG	TATGGCTGCT	1320
TGTGGGCGTT	TCGCACTAAG	AAAGTACATT	CGTTTTATCG	CCCACCTGGA	ACGCAGACCT	1380
GCGTAAAAGT	CCCAGCCTCT	TTTAGCGCTT	TTCCCATGTC	GTCCGTATGG	ACGACCTCTT	1440
TGCCCATGTC	GCTGAGGCAG	AAATTGAAAC	TGGCATTGCA	ACCAAGAAG	GAGGAAAAAC	1500
TGCTGCAGGT	CTCGGAGGAA	TTAGTCATGG	AGGCCAAGGC	TGCTTTTGAG	GATGCTCAGG	1560
AGGAAGCCAG	AGCGGAGAAG	CTCCGAGAAG	CACTTCCACC	ATTAGTGGCA	GACAAAGGCA	1620
TCGAGGCAGC	CGCAGAAGTT	GTCTGCGAAG	TGGAGGGGCT	CCAGGCGGAC	ATCGGAGCAG	1680
CATTAGTTGA	AACCCCGCGC	GGTCACGTAA	GGATAATACC	TCAAGCAAA	GACCGTATGA	1740
TCGGACAGTA	TATCGTTGTC	TCGCCAAACT	CTGTGCTGAA	GAATGCCAAA	CTCGCACCAG	1800
CGCACCCGCT	AGCAGATCAG	GTTAAGATCA	TAACACACTC	CGGAAGATCA	GGAAGGTACG	1860
CGGTGGAACC	ATACGACGCT	AAAGTACTGA	TGCCAGCAGG	AGGTGCCGTA	CCATGGCCAG	1920
AATTCCTAGC	ACTGAGTGAG	AGCGCCACGT	TAGTGTACAA	CGAAAGAGAG	TTTGTGAACC	1980
GCAAACTATA	CCACATTGCC	ATGCATGGCC	CCGCCAAGAA	TACAGAAGAG	GAGCAGTACA	2040
AGGTTACAAA	GGCAGAGCTT	GCAGAAACAG	AGTACGTGTT	TGACGTGGAC	AAGAAGCGTT	2100
GCGTTAAGAA	GGAAGAAGCC	TCAGGTCTGG	TCCTCTCGGG	AGAACTGACC	AACCTCCCT	2160
ATCATGAGCT	AGCTCTGGAG	GGACTGAAGA	CCCGACCTGC	GGTCCCGTAC	AAGGTGCAAA	2220
CAATAGGAGT	GATAGGCACA	CCGGGGTCGG	GCAAGTCAGC	TATTATCAAG	TCAACTGTCA	2280
CGGCACGAGA	TCTTGTTACC	AGCGGAAAGA	AAGAAAATTG	TCGCGAAATT	GAGGCCGACG	2340
TGCTAAGACT	GAGGGGTATG	CAGATTACGT	CGAAGACAGT	AGATTCCGTT	ATGCTCAACG	2400
GATGCCACAA	AGCCGTAGAA	GTGCTGTACG	TTGACGAAGC	GTTCCGCTGC	CACGCAGGAG	2460
CACTACTTGC	CTTGATTGCT	ATCGTCAGGC	CCCGCAAGAA	GGTAGTACTA	TGCGGAGACC	2520

*Fig. 7A*

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TCAGAGGGGA	AATAAAGCAT	CTCTACGGTG	GTCCTAAATA	GTCAGCATAG	TTCATTTTCAT	7620
CTGACTAATA	CTACAACACC	ACCACCATGA	ATAGAGGATT	CTTTAACATG	CTCGGCCGCC	7680
GCCCCCTCCC	GGCCCCCACT	GCCATGTGGA	GGCCGCGGAG	AAGGAGGCAG	GCGGCCCCGA	7740
TGCCTGCCCC	CAACGGGCTG	GCTTCTCAAA	TCCAGCAACT	GACCACAGCC	GTCAGTGCCC	7800
TAGTCATTGG	ACAGGCAACT	AGACCTCAAC	CCCCATGTCC	ACGCCCCGCCA	CCGCGCCAGA	7860
AGAAGCAGGC	GCCCAAGCAA	CCACCGAAGC	CGAAGAAACC	AAAAACGCAG	GAGAAGAAGA	7920
AGAAGCAACC	TGCAAAACCC	AAACCCGGAA	AGAGACAGCG	CATGGCACTT	AAGTTGGAGG	7980
CCGACAGATT	GTTGACGTC					8000

*Fig. 6D*

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CGCACACTCC	CGCATTTCGT	CCCGCCCGTA	AGTACATAGA	AGTGCCAGAA	CAGCCTACCG	5100
CTCCTCCTGC	ACAGGCCGAG	GAGGCCCGCG	AAGTTGTAGC	GACACCGTCA	CCATCTACAG	5160
CTGATAACAC	CTCGCTTGAT	GTCACAGACA	TCTCACTGGA	TATGGATGAC	AGTAGCGAAG	5220
GCTCACTTTT	TTCGAGCTTT	AGCGGATCGG	ACAACTCTAT	TACTAGTATG	GACAGTTGGT	5280
CGTCAGGACC	TAGTTCACTA	GAGATAGTAG	ACCGAAGGCA	GGTGGTGGTG	GCTGACGTTT	5340
ATGCCGTCCA	AGAGCCTGCC	CCTATTCCAC	CGCCAAGGCT	AAAGAAGATG	GCCCGCCTGG	5400
CAGCGGCAAG	AAAAGAGCCC	ACTCCACCGG	CAAGCAATAG	CTCTGAGTCC	CTCCACCTCT	5460
CTTTTGGTGG	GGTATCCATG	TCCCTCGGAT	CAATTTTCGA	CGGAGAGACG	GCCCGCCAGG	5520
CAGCGGTACA	ACCCCTGGCA	ACAGGCCCCA	CGGATGTGCC	TATGTCTTTC	GGATCGTTTT	5580
CCGACGGAGA	GATTGATGAG	CTGAGCCGCA	GAGTAACTGA	GTCCGAACCC	GTCCTGTTTG	5640
GATCATTTGA	ACCGGGCGAA	GTGAACTCAA	TTATATCGTC	CCGATCAGCC	GTATCTTTTC	5700
CTCTACGCAA	GCAGAGACGT	AGACGCAGGA	GCAGGAGGAC	TGAATACTGA	CTAACCGGGG	5760
TAGGTGGGTA	CATATTTTCG	ACGGACACAG	GCCCTGGGCA	CTTGCAAAAG	AAGTCCGTTC	5820
TGCAGAACCA	GCTTACAGAA	CCGACCTTGG	AGCACAATGT	CCTGGAAAGA	ATTATGCCC	5880
CGGTGCTCGA	CACGTGCGAA	GAGGAACAAC	TCAAACCTAG	GTACCAGATG	ATGCCACCCG	5940
AAGCCAACAA	AAGTAGGTAC	CAGTCTCGTA	AAGTAGAAAA	TCAGAAAGCC	ATAACCACTG	6000
AGCGACTACT	GTCAGGACTA	CGACTGTATA	ACTCTGCCAC	AGATCAGCCA	GAATGCTATA	6060
AGATCACCTA	TCCGAAACCA	TTGTACTCCA	GTAGCGTACC	GGCGAACTAC	TCCGATCCAC	6120
AGTTCCGTGT	AGCTGTCTGT	AACAACATATC	TGCATGAGAA	CTATCCGACA	GTAGCATCTT	6180
ATCAGATTAC	TGACGAGTAC	GATGCTTACT	TGGATATGGT	AGACGGGACA	GTCGCCCTGCC	6240
TGGATACTGC	AACCTTCTGC	CCCGCTAAGC	TTAGAAGTTA	CCCGAAAAAA	CATGAGTATA	6300
GAGCCCCGAA	TATCCGCAGT	GCGGTTCAT	CAGCGATGCA	GAACACGCTA	CAAAATGTGC	6360
TCATTGCCGC	AACTAAAAGA	AATTGCAACG	TCACGCAGAT	GCGTGAAGTG	CCAACACTGG	6420
ACTCAGCGAC	ATTCAATGTC	GAATGCTTTC	GAAAATATGC	ATGTAATGAC	GAGTATTGGG	6480
AGGAGTTCCG	TCGGAAGCCA	ATTAGGATTA	CCACTGAGTT	TGTCACCGCA	TATGTAGCTA	6540
GACTGAAAGG	CCCTAAGGCC	GCCGCACTAT	TTGCAAAGAC	GTATAATTTG	GTCCCATTCG	6600
AAGAAGTGCC	TATGGATAGA	TTCGTCATGG	ACATGAAAAG	AGACGTGAAA	GTTACACCAAG	6660
GCACGAAACA	CACAGAAGAA	AGACCGAAAG	TACAAGTGAT	ACAAGCCGCA	GAACCCCTGG	6720
CGACTGCTTA	CTTATGCGGG	ATTACCCGGG	AATTAGTGCG	TAGGCTTACG	GCCGTCTTGC	6780
TTCCAAACAT	TCACACGCTT	TTTGACATGT	CGGCGGAGGA	TTTGTATGCA	ATCATAGCAG	6840
AACACTTCAA	GCAAGGCGAC	CCGGTACTGG	AGACGGATAT	CGCATCATTC	GACAAAAGCC	6900
AAGACGACGC	TATGGCGTTA	ACCGGTCTGA	TGATCTTGGA	GGACCTGGGT	GTGGATCAAC	6960
CACTACTCGA	CTTGATCGAG	TGCGCCTTTG	GAGAAATATC	ATCCACCCAT	CTACCTACGG	7020
GTA CTGTTT	TAAATTGCGG	GCGATGATGA	AATCCGGAAT	GTTCTCACA	CTTTTGTCA	7080
ACACAGTTTT	GAATGTCGTT	ATCGCCAGCA	GAGTACTAGA	AGAGCGGCTT	AAAACGTCCA	7140
GATGTGCAGC	GTTCAATTGGC	GACGACAACA	TCATACATGG	AGTAGTATCT	GACAAAGAAA	7200
TGGCTGAGAG	GTGCGCCACC	TGGCTCAACA	TGGAGGTTAA	GATCATCGAC	GCAGTCATCG	7260
GTGAGAGACC	ACCTTACTTC	TGCGGCGGAT	TTATCTTGCA	AGATTCCGTT	ACTTCCACAG	7320
CGTGCCGCGT	GGCGGATCCC	CTGAAAAGGC	TGTTTAAGTT	GGGTAAACCG	CTCCACGCCG	7380
ACGACGAGCA	AGACGAAGAC	AGAAGACGCG	CTCTGCTAGA	TGAAACAAAG	GCGTGGTTTA	7440
GAGTAGGTAT	AACAGGCACT	TTAGCAGTGG	CCGTGACGAC	CCGGTATGAG	GTAGACAATA	7500
TTACACCTGT	CCTACTGGCA	TTGAGAAGTT	TTGCCAGAG	CAAAAGAGCA	TTCCAAGCCA	7560

*Fig. 6C*

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CCATGCAATG	CGGATTCTTC	AACATGATGC	AACTAAAGGT	ACATTTCAAT	CACCCTGAAA	2580
AAGACATATG	CACCAAGACA	TTCTACAAGT	ATATCTCCCG	GCGTTGCACA	CAGCCAGTTA	2640
CAGCTATTGT	ATCGACACTG	CATTACGATG	GAAAGATGAA	AACCACGAAC	CCGTGCAAGA	2700
AGAACATTGA	AATCGATATT	ACAGGGGCCA	CAAAGCCGAA	GCCAGGGGAT	ATCATCCTGA	2760
CATGTTTCCG	CGGGTGGGTT	AAGCAATTGC	AAATCGACTA	TCCCGGACAT	GAAGTAATGA	2820
CAGCCGCGGC	CTCACAAGGG	CTAACCAGAA	AAGGAGTGTA	TGCCGTCCGG	CAAAAAGTCA	2880
ATGAAAACCC	ACTGTACGCG	ATCACATCAG	AGCATGTGAA	CGTGTGCTC	ACCCGCACTG	2940
AGGACAGGCT	AGTGTGGAAA	ACCTTGCAGG	GCGACCCATG	GATTAAGCAG	CTACTAACA	3000
TACCTAAAGG	AAACTTTTCA	GCTACTATAG	AGGACTGGGA	AGCTGAACAC	AAGGGAATAA	3060
TTGCTGCAAT	AAACAGCCCC	ACTCCCCGTG	CCAATCCGTT	CAGCTGCAAG	ACCAACGTTT	3120
GCTGGGCGAA	AGCATTGGAA	CCGATACTAG	CCACGGCCGG	TATCGTACTT	ACCGGTTGCC	3180
AGTGGAGCGA	ACTGTTCCCA	CAGTTTGCGG	ATGACAAACC	ACATTCGGCC	ATTTACGCCT	3240
TAGACGTAAT	TTGCATTAA	TTTTTCGGCA	TGGACTTGAC	AAGCGGACTG	TTTTCTAAAC	3300
AGAGCATCCC	ACTAACGTAC	CATCCCGCCG	ATTCAGCGAG	GCCGGTAGCT	CATTGGGACA	3360
ACAGCCCAGG	AACCCGCAAG	TATGGGTACG	ATCACGCCAT	TGCCGCCGAA	CTCTCCCGTA	3420
GATTTCCGGT	GTTCCAGCTA	GCTGGGAAGG	GCACACAAC	TGATTTGCAG	ACGGGGAGAA	3480
CCAGAGTTAT	CTCTGCACAG	CATAACCTGG	TCCCGGTGAA	CCGCAATCTT	CCTCACGCCT	3540
TAGTCCCCGA	GTACAAGGAG	AAGCAACCCG	GCCCGGTGCA	AAAATTCTTG	AACCAGTTCA	3600
AACACCACTC	AGTACTTGTT	GTATCAGAGG	AAAAAATTGA	AGTCCCCCGT	AAGAGAATCG	3660
AATGGATCGC	CCCGATTGGC	ATAGCCGGTG	CAGATAAGAA	CTACAACCTG	GCTTTCGGGT	3720
TTCCGCCGCA	GGCACGGTAC	GACCTGGTGT	TCATCAACAT	TGGAACATAA	TACAGAAACC	3780
ACCACTTTCA	GCAGTGCGAA	GACCATGCGG	CGACCTTAAA	AACCCTTTCG	CGTTCGGCCC	3840
TGAATTGCCT	TAACTCAGGA	GGCACTCTCG	TGGTGAAGTC	CTATGGCTAC	GCCGACCGCA	3900
ACAGTGAGGA	CGTAGTCACC	GCTCTTGCCA	GAAAGTTTGT	CAGGGTGTCT	GCAGCGAGAC	3960
CAGATTGTGT	CTCAAGCAAT	ACAGAAATGT	ACCTGATTTT	CCGACAATA	GACAACAGCC	4020
GTACACGGCA	ATTCACCCCG	CACCATCTGA	ATTGCGTGAT	TTCGTCCGTG	TATGAGGGTA	4080
CAAGAGATGG	AGTTGGAGCC	GCGCCGTGAT	ACCGCACCAA	AAGGGAGAAT	ATTGCTGACT	4140
GTCAAGAGGA	AGCAGTTGTC	AACGCAGCCA	ATCCGCTGGG	TAGACCAGGC	GAAGGAGTCT	4200
GCCGTGCCAT	CTATAAACGT	TGGCCGACCA	GTTTTACCGA	TTCAGCCACG	GAGACAGGCA	4260
CCGCAAGAAT	GACTGTGTGC	CTAGGAAAGA	AAGTGATCCA	CGCGGTCGGC	CCTGATTTC	4320
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ACTTAGTAAA	TGAACATAAC	ATCAAGTCTG	TCGCCATTCC	ACTGCTATCT	ACAGGCATTT	4440
ACGCAGCCGG	AAAAGACCGC	CTTGAAGTAT	CACTTAACTG	CTTGACAACC	GCGCTAGACA	4500
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CGGCACTCCA	ACTTAAGGAG	TCTGTAACAG	AGCTGAAGGA	TGAAGATATG	GAGATCGACG	4620
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CAAAAGGAAA	ATTGTATTCT	TACTTCGAAG	GCACCAAAAT	CCATCAAGCA	GCAAAAGACA	4740
TGGCGGAGAT	AAAGGTCCTG	TTCCCTAATG	ACCAGGAAAG	TAATGAACAA	CTGTGTGCCT	4800
ACATATTGGG	TGAGACCATG	GAAGCAATCC	GCGAAAAGTG	CCCGGTCGAC	CATAACCCGT	4860
CGTTTAGCCC	GCCCAAAACG	TTGCCGTGCC	TTTGCAATGA	TGCCATGACG	CCAGAAAGGG	4920
TCCACAGACT	TAGAAGCAAT	AACGTCAAAG	AAGTTACAGT	ATGCTCCTCC	ACCCCCCTTC	4980
CTAAGCACAA	AATTAAGAAT	GTTCAGAAGG	TTCAGTGCAC	GAAAGTAGTC	CTGTTTAATC	5040

*Fig. 6B*

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ATTGACGGCG	TAGTACACAC	TATTGAATCA	AACAGCCGAC	CAATCGCACT	ACCATCACAA	60
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AAAAAAGCTT	CCCGCAATT	GAGGTAGTAG	CACAGCAGGT	CACTCCAAAT	GACCATGCTA	180
ATGCCAGAGC	ATTTTCGCAT	CTGGCCAGTA	AACTAATCGA	GCTGGAGGTT	CCTACCACAG	240
CGACGATCTT	GGACATAGGC	AGCGCACC GG	CTCGTAGAAT	GTTTTCCGAG	CACCAGTATC	300
ATTGTGTCTG	CCCCATGCGT	AGTCCAGAAG	ACCCGGACCG	CATGATGAAA	TACGCCAGTA	360
AACTGGCGGA	AAAAGCGTGC	AAGATTACAA	ACAAGA ACTT	GCATGAGAAG	ATTAAGGATC	420
TCCGGACCGT	ACTTGATACG	CCGGATGCTG	AAACACCATC	GCTCTGCTTT	CACAACGATG	480
TTACCTGCAA	CATGCGTGCC	GAATATTCCG	TCATGCAGGA	CGTGTATATC	AACGCTCCCG	540
GAACTATCTA	TCATCAGGCT	ATGAAAGGCG	TGCGGACCCT	GTA CTGGATT	GGCTTCGACA	600
CCACCCAGTT	CATGTTCTCG	GCTATGGCAG	GTTCTGTACCC	TGCGTACAAC	ACCAACTGGG	660
CCGACGAGAA	AGTCCTTGAA	GCGCGTAACA	TCGGACTTTG	CAGCACAAAG	CTGAGTGAAG	720
GTAGGACAGG	AAAATTGTCTG	ATAATGAGGA	AGAAGGAGTT	GAAGCCCGGG	TCGCGGGTTT	780
ATTTCTCCGT	AGGATCGACA	CTTTATCCAG	AACACAGAGC	CAGCTTGCA G	AGCTGGCATC	840
TTCCATCGGT	GTTCCACTTG	AATGGAAAGC	AGTCGTACAC	TTGCCGCTGT	GATACAGTGG	900
TGAGTTGCGA	AGGCTACGTA	GTGAAGAAAA	TCACCATCAG	TCCCGGGATC	ACGGGAGAAA	960
CCGTGGGATA	CGCGGTTACA	CACAATAGCG	AGGGCTTCTT	GCTATGCAAA	GTTACTGACA	1020
CAGTAAAAGG	AGAACGGGTA	TCGTTCCCTG	TGTGCACGTA	CATCCCGGCC	ACCATATGCG	1080
ATCAGATGAC	TGGTATAATG	GCCACGGATA	TATCACCTGA	CGATGCACAA	AAACTTCTGG	1140
TTGGGCTCAA	CCAGCGAATT	GTCATTAACG	GTAGGACTAA	CAGGAACACC	AACACCATGC	1200
AAAATTACCT	TCTGCCGATC	ATAGCACAA G	GGTTCAGCAA	ATGGGCTAAG	GAGCGCAAGG	1260
ATGATCTTGA	TAACGAGAAA	ATGCTGGGTA	CTAGAGAACG	CAAGCTTACG	TATGGCTGCT	1320
TGTGGGCGTT	TCGCACTAAG	AAAGTACATT	CGTTTTATCG	CCCACCTGGA	ACGCAGACCT	1380
GCGTAAAAGT	CCCAGCCTCT	TTAGCGCTT	TTCCCATGTC	GTCCGTATGG	ACGACCTCTT	1440
TGCCCATGTC	GCTGAGGCAG	AAATTGAAAC	TGGCATTGCA	ACCAAGAAG	GAGGAAAAAC	1500
TGCTGCAGGT	CTCGGAGGAA	TTAGTCATGG	AGGCCAAGGC	TGCTTTTGAG	GATGCTCAGG	1560
AGGAAGCCAG	AGCGGAGAAG	CTCCGAGAAG	CACTTCCACC	ATTAGTGGCA	GACAAAGGCA	1620
TCGAGGCAGC	CGCAGAAGTT	GTCTGCCAAG	TGGAGGGGCT	CCAGGCGGAC	ATCGGAGCAG	1680
CATTAGTTGA	AACCCCGCGC	GGTCACGTAA	GGATAATACC	TCAAGCAAAT	GACCGTATGA	1740
TCGGACAGTA	TATCGTTGTC	TCGCCAAACT	CTGTACTGAA	GAATGCCAAA	CTCGCACCAG	1800
CGCACCCGCT	AGCAGATCAG	GTTAAGATCA	TAACACACTC	CGGAAGATCA	GGAAGGTACG	1860
CGGTGGAACC	ATACGACGCT	AAAGTACTGA	TGCCAGCAGG	AGGTGCCGTA	CCATGCCCAG	1920
AATTCCTAGC	ACTGAGTGAG	AGCGCCACGT	TAGTGTACAA	CGAAAGAGAG	CTTGTGAACC	1980
GCAAACTATA	CCACATTGCC	ATGCATGGCC	CCGCCAAGAA	TACAGAAGAG	GAGCAGTACA	2040
AGGTTACAAA	GGCAGAGCTT	GCAGAAACAG	AGTACGTGTT	TGACGTGGAC	AAGAAGCGTT	2100
GCGTTAAGAA	GGAAGAAGCC	TCAGGTCTGG	TCCTCTCGGG	AGAACTGACC	AACCCTCCCT	2160
ATCATGAGCT	AGCTCTGGAG	GGACTGAAGA	CCCGACCTGC	GGTCCCGTAC	AAGGTGGAAG	2220
CAATAGGAGT	GATAGGCACA	CCGGGGTCCG	GCAAGTCAGC	TATTATCAAG	TCAACTGTCA	2280
CGGCACGAGA	TCTTGTTACC	AGCGGAAAGA	AAGAAAATTG	TCGCGAAATT	GAGGCCGACG	2340
TGCTAAGACT	GAGGGGTATG	CAGATTACGT	CGAAGACAGT	AGATTCGGTT	ATGCTCAACG	2400
GATGCCACAA	AGCCGTAGAA	GTGCTGTACG	TTGACGAAGC	GTTCCGCTGC	CACGCAGGAG	2460
CACTACTTGC	CTTGATTGCT	ATCGTCAGGC	CCCGCAAGAA	GGTAGTACTA	TGCGGAGACC	2520

*Fig. 6A*

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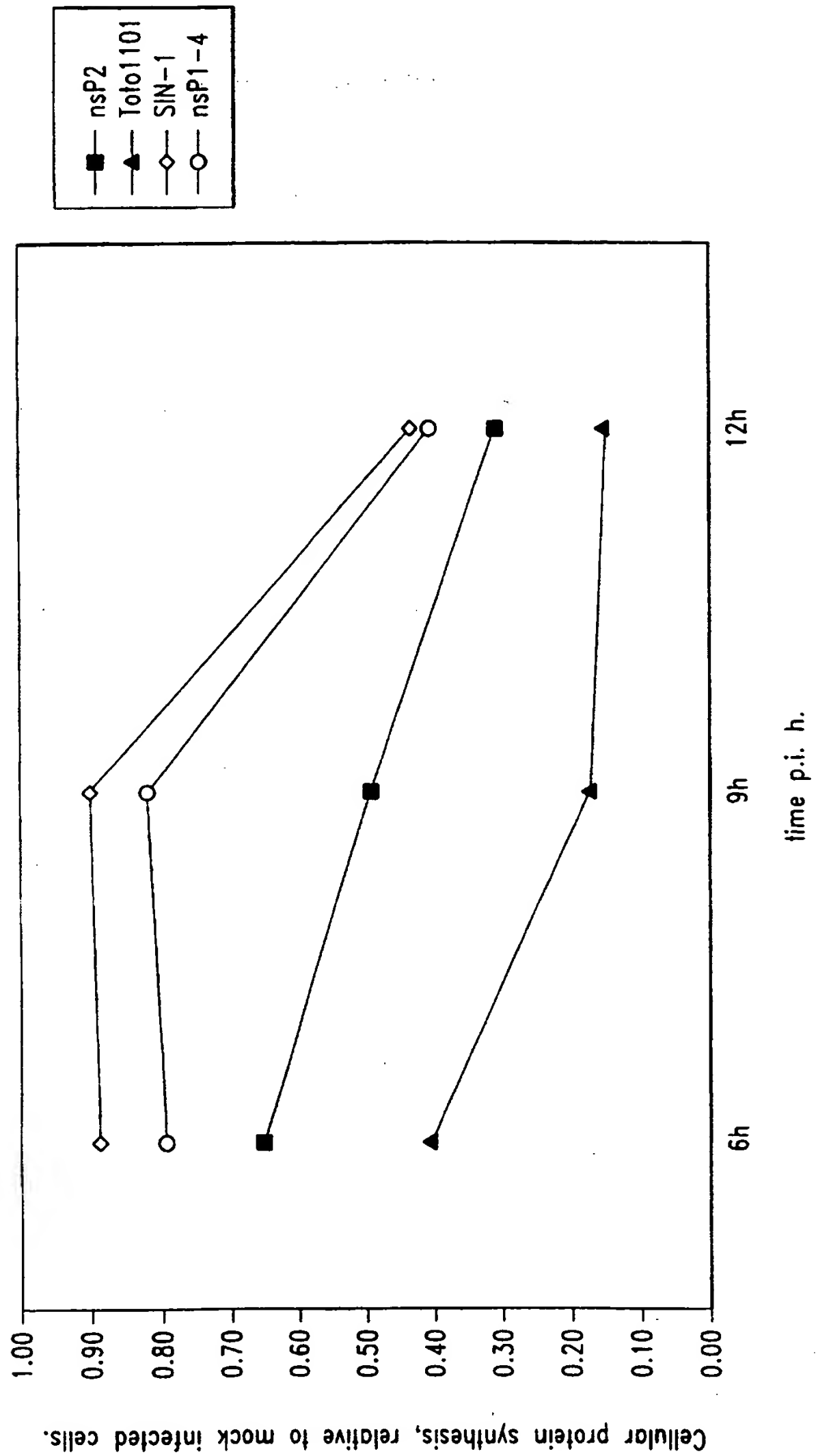
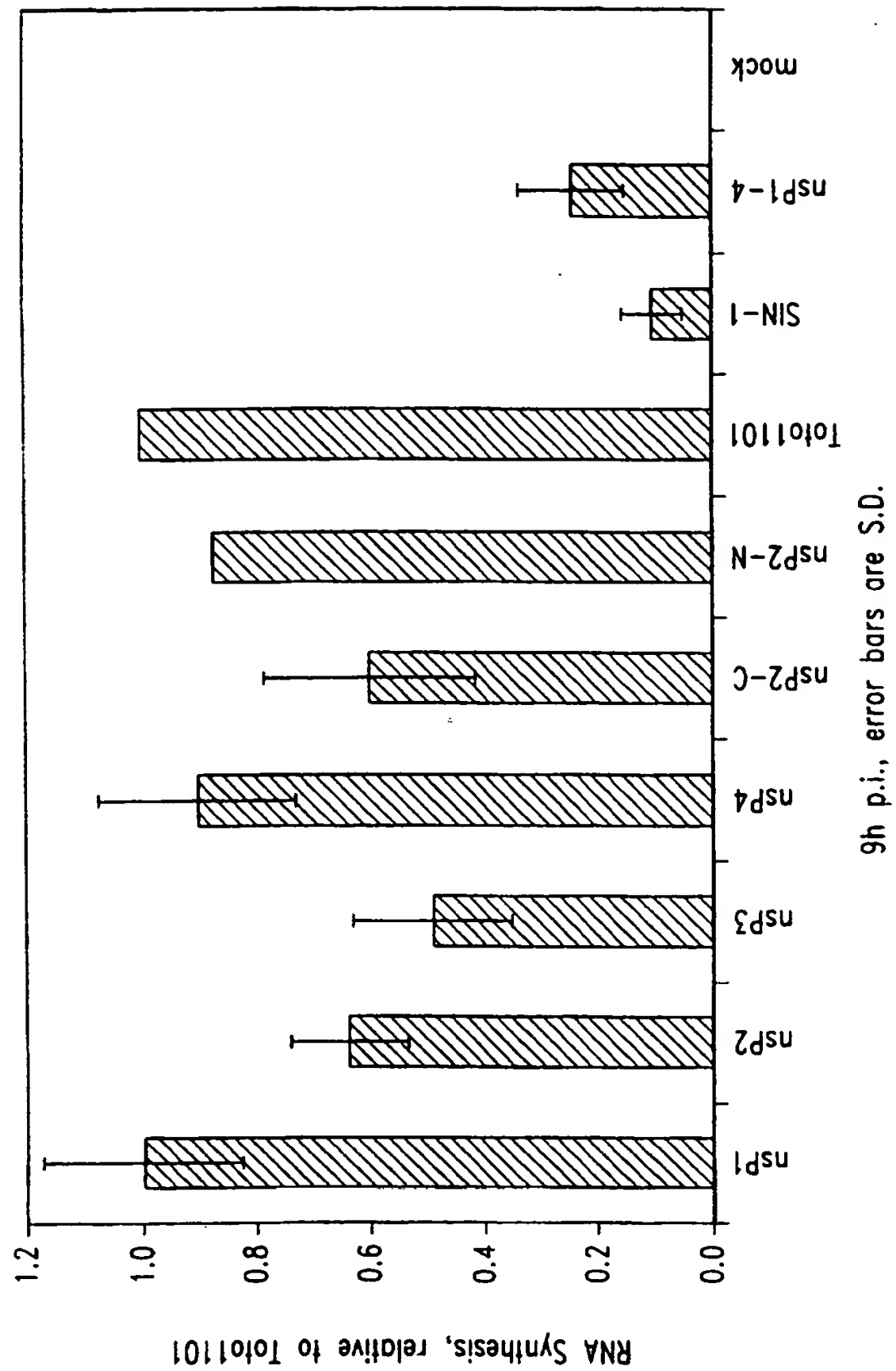


Fig. 5

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*Fig. 4*

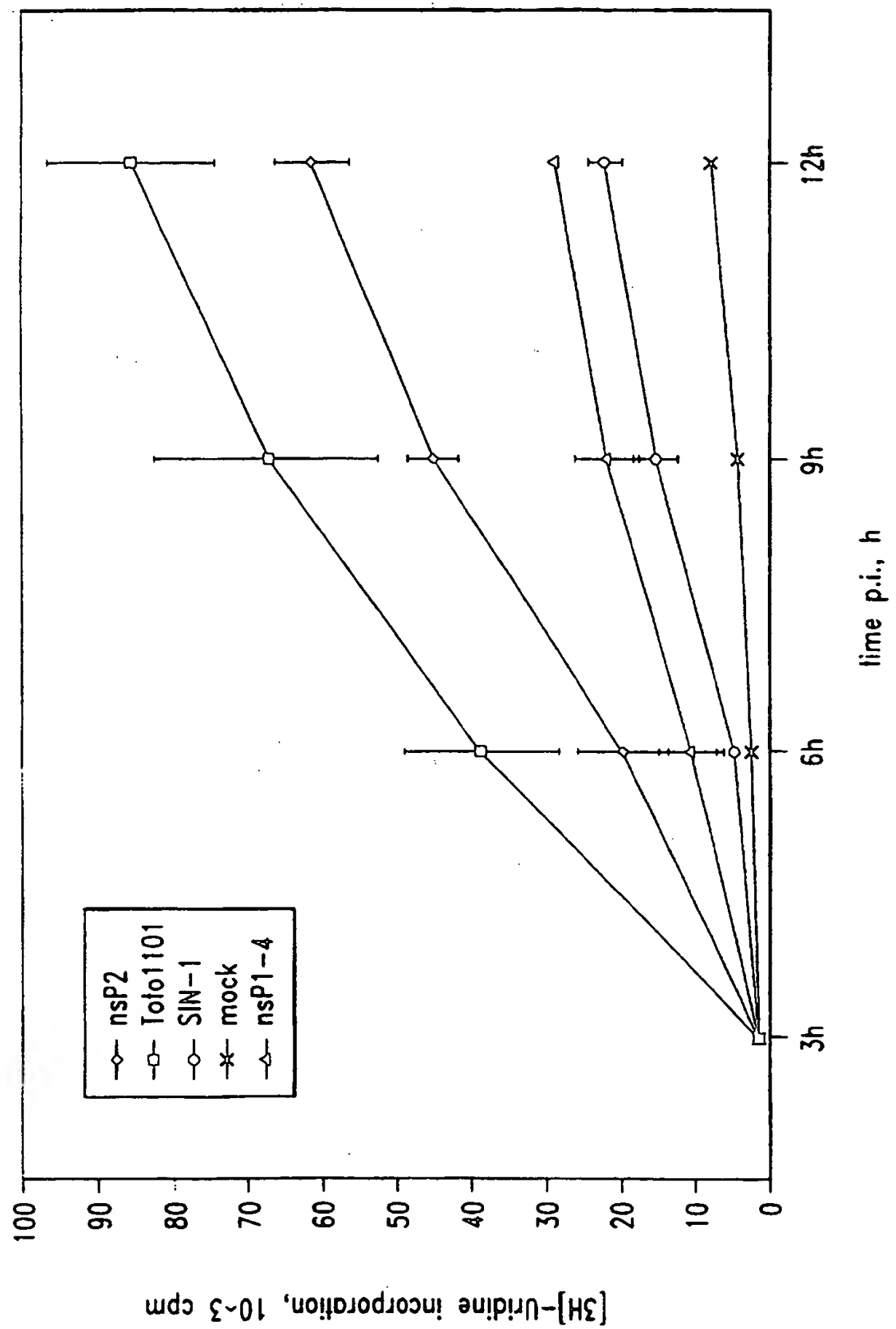


Fig. 3

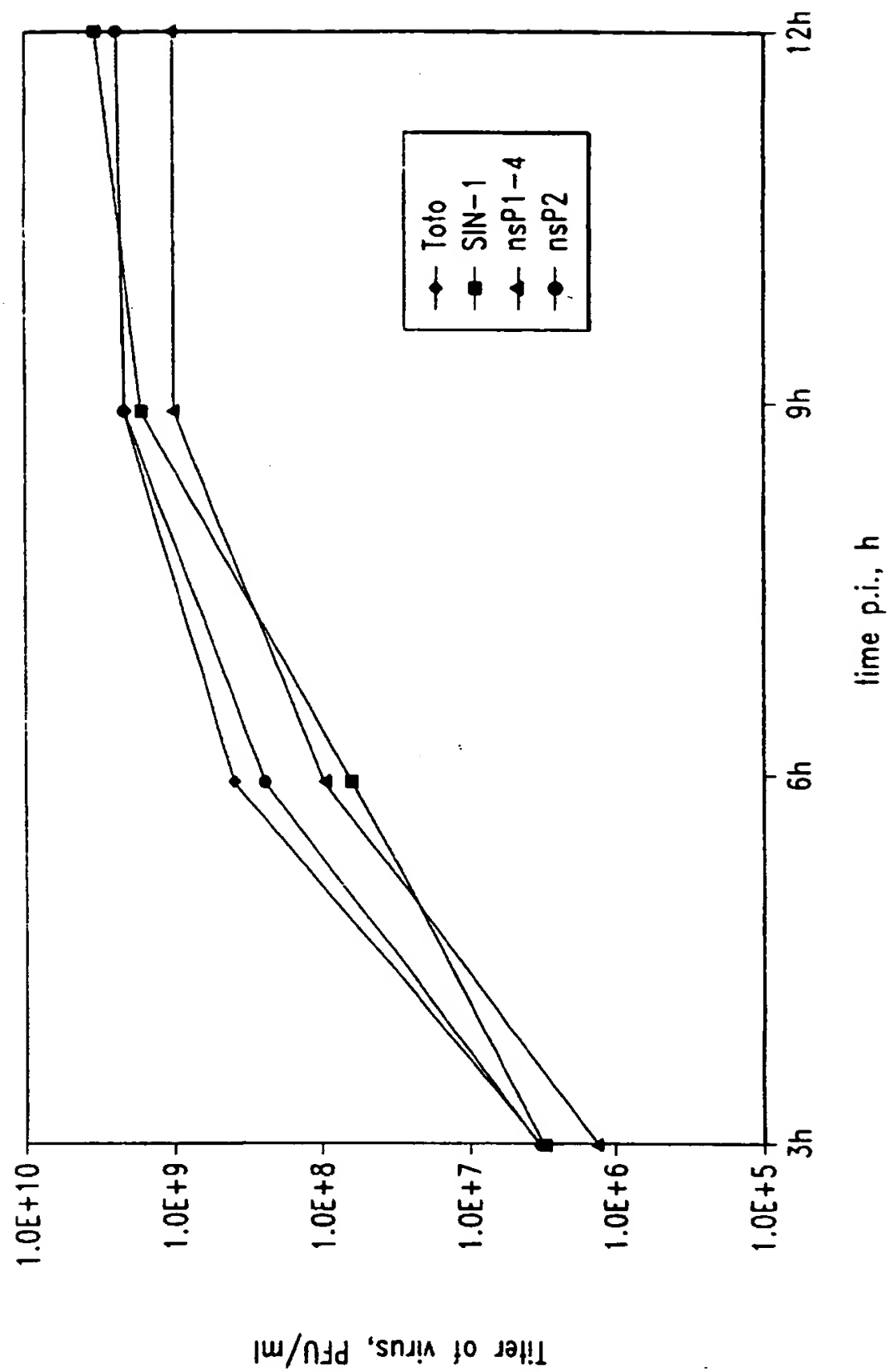
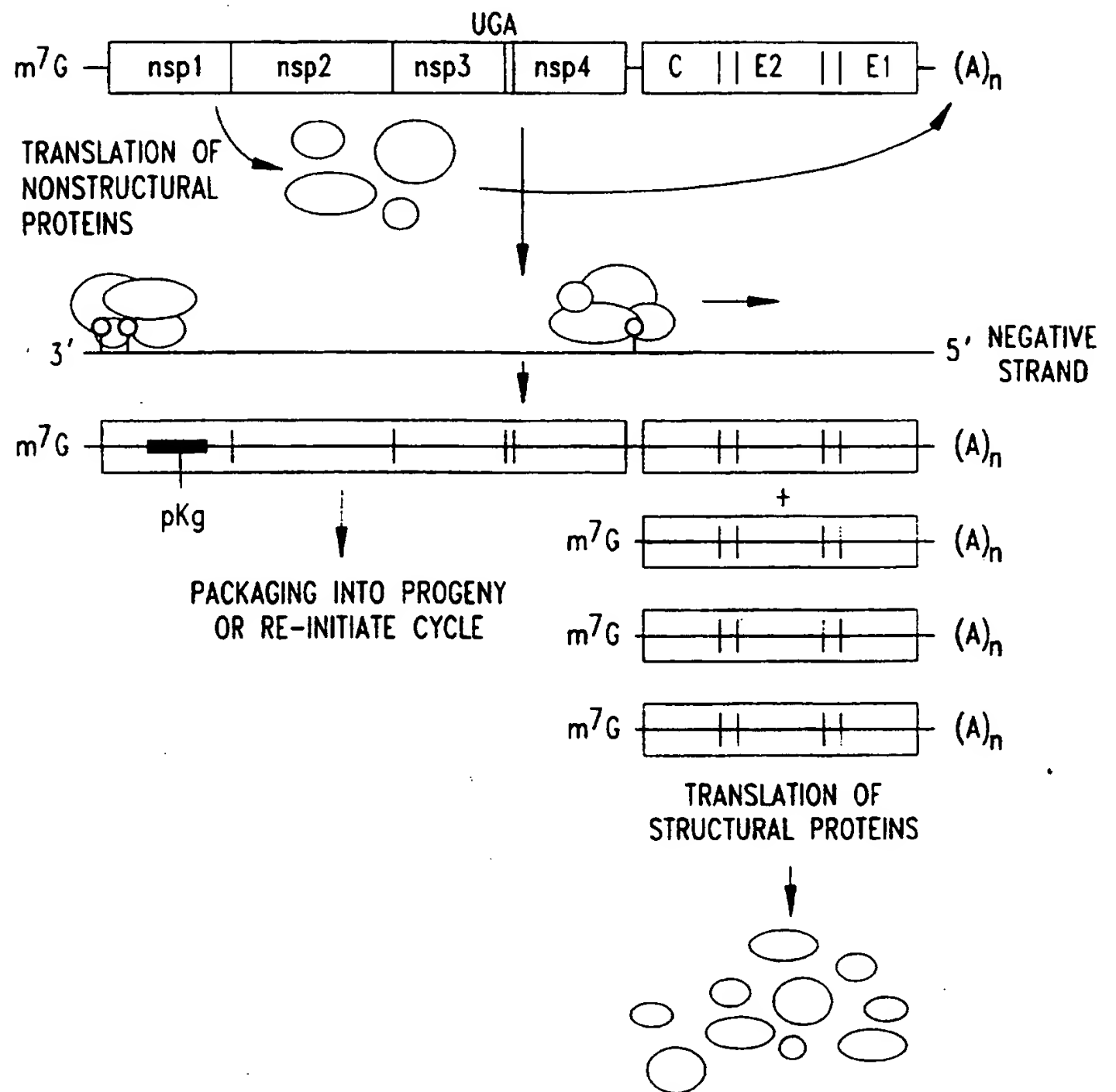


Fig. 2



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## SINDBIS GENOME ORGANIZATION AND REPLICATION STRATEGY

*Fig. 1*

recombinant alphavirus particle according to claim 6, or a eukaryotic layered vector initiation system according to claim 18.

22. A method of making recombinant alphavirus particles, comprising:

- (a) introducing a vector selected from the group consisting of a eukaryotic layered vector initiation system according to claim 18, an RNA vector replicon according to claim 4, and a recombinant alphavirus vector particle according to claim 6, into a population of packaging cells according to claim 16, under conditions and for a time sufficient to permit production of recombinant alphavirus particles; and
- (b) harvesting recombinant alphavirus particles.

23. A method of making a selected protein, comprising:

- (a) introducing a vector which encodes a selected heterologous protein, and which is selected from the group consisting of a eukaryotic layered vector initiation system according to claim 18, an alphavirus RNA vector replicon according to claim 4, and a recombinant alphavirus vector particle according to claim 6, into a population of packaging cells according to claim 16, under conditions and for a time sufficient to permit production or said selected protein; and
- (b) harvesting protein produced by the packaging cells.

24. A method of making a selected protein, comprising introducing a eukaryotic layered vector initiation system according to claim 18 into a host cell, under conditions and for sufficient to permit expression of said selected protein.

25. A host cell line which contains an alphavirus RNA vector replicon according to claim 4.

encodes one or more functional alphavirus structural proteins, a selectable marker operably linked to transcription of the expression cassette, and a 3' sequence which controls transcription termination.

16. An alphavirus packaging cell line, comprising a cell containing an alphavirus structural protein expression cassette according to claim 15.

17. An alphavirus producer cell line, comprising a cell containing a stably transformed alphavirus structural protein expression cassette, and a vector selected from the group consisting of an RNA vector replicon according to claim 4, an alphavirus vector construct according to claim 3, and a eukaryotic layered vector initiation system according to claim 18.

18. A eukaryotic layered vector initiation system, comprising a 5' promoter capable of initiating *in vivo* the 5' synthesis of alphavirus RNA from cDNA, a sequence which initiates transcription of alphavirus RNA following the 5' promoter, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, including a nucleic acid molecule according to claims 1 or 2, an alphavirus RNA polymerase recognition sequence, and a 3' polyadenylate tract.

19. A host cell containing a eukaryotic layered vector initiation system according to claim 18.

20. A pharmaceutical composition, comprising a eukaryotic layered vector initiation system according to claim 18 and a pharmaceutically acceptable carrier or diluent.

21. A method for delivering a selected heterologous sequence to a vertebrate or insect, comprising administering to a vertebrate or insect an alphavirus vector construct according to claim 3, an alphavirus RNA vector replicon according to claim 4, a

6. A recombinant alphavirus particle, comprising one or more alphavirus structural proteins, a lipid envelope, and an RNA vector replicon according to claim 4.
7. The recombinant alphavirus particle according to claim 6 wherein said alphavirus structural protein and lipid envelope are derived from different alphavirus species.
8. A pharmaceutical composition, comprising a recombinant alphavirus particle according to claim 6 or 7 and a pharmaceutically acceptable carrier or diluent.
9. A host cell infected with a recombinant alphavirus particle according to claim 6 or 7.
10. A togavirus capsid particle which contains substantially no genomic or RNA Vector Replicon nucleic acids.
11. The capsid particle according to claim 10, further comprising a lipid envelope containing one or more alphavirus glycoproteins.
12. The capsid particle according to claim 10, further comprising an alphavirus envelope.
13. The capsid particle according to claim 10 wherein said capsid is derived from a togavirus selected from the group consisting of alphaviruses, rubiviruses, flaviviruses and pestiviruses.
14. A pharmaceutical composition, comprising a capsid particle according to any one of claims 10 to 13, and a pharmaceutically acceptable carrier or diluent.
15. An alphavirus structural protein expression cassette, comprising a 5' promoter which initiates synthesis of RNA from DNA, a nucleic acid molecule which

Claims

We claim:

1. An isolated nucleic acid molecule, comprising an altered alphavirus nonstructural protein gene which, when operably incorporated into a recombinant alphavirus particle, increases the time required to reach 50% inhibition of host-cell directed macromolecular synthesis following expression in mammalian cells, as compared to a wild-type alphavirus.
2. An isolated nucleic acid molecule, comprising an alphavirus nonstructural protein gene which, when operably incorporated into a recombinant alphavirus particle, has a reduced level of vector-specific RNA synthesis, as compared to the wild-type, and the same or greater level of proteins encoded by RNA transcribed from the viral junction region promoter, as compared to a wild-type recombinant alphavirus particle.
3. An alphavirus vector construct, comprising a 5' promoter which initiates synthesis of viral RNA *in vitro* from cDNA, a 5' sequence which initiates transcription of alphavirus RNA, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins including a nucleic acid molecule according to claims 1 or 2, an alphavirus RNA polymerase recognition sequence and a 3' polyadenylate tract.
4. An alphavirus RNA vector replicon capable of translation in a eukaryotic system, comprising a 5' sequence which initiates transcription of alphavirus RNA, a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, including a nucleic acid molecule according to claims 1 or 2, an alphavirus RNA polymerase recognition sequence and a 3' polyadenylate tract.
5. A pharmaceutical composition, comprising an alphavirus RNA vector replicon according to claim 4 and a pharmaceutically acceptable carrier or diluent.

255

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

ATATAGGATC CTTCGCATGA TTGAACAAGA TGGATTGC

38

Lys Ala Pro Lys Gly Gln Arg Gln Lys Lys Gln Leu Lys Arg Arg Glu  
 65 70 75 80

Arg Met Cys Met Lys Ile Glu Asn  
 85

(2) INFORMATION FOR SEQ ID NO:117:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

Met Asn Tyr Ile Pro Thr Gln Thr Phe Tyr Gly Arg Arg Trp Arg Pro  
 1 5 10 15

Arg Pro Ala Phe Arg Pro Trp Gln Val Ser Met Gln Pro Thr Pro Thr  
 20 25 30

Met Val Thr Pro Met Leu Gln Ala Pro Asp Leu Gln Ala Gln Gln Met  
 35 40 45

Gln Gln Leu Ile Ser Ala Val Ser Ala Leu Thr Thr Lys Gln Asn Leu  
 50 55 60

Lys Arg Arg Glu Arg Met Cys Met Lys Ile Glu Asn  
 65 70 75

(2) INFORMATION FOR SEQ ID NO:118:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

253

1                      5                      10                      15  
 Arg Pro Ala Phe Arg Pro Trp Gln Val Ser Met Gln Pro Thr Pro Thr  
                          20                                      25                                      30  
 Met Val Thr Pro Met Leu Gln Ala Pro Asp Leu Gln Ala Gln Gln Met  
                          35                                      40                                      45  
 Gln Gln Leu Ile Ser Ala Val Ser Ala Leu Thr Thr Lys Gln Asn Val  
                          50                                      55                                      60  
 Lys Ala Pro Lys Gly Gln Arg Gln Lys Lys Gln Gln Lys Pro Lys Glu  
                          65                                      70                                      75                                      80  
 Lys Lys Glu Asn Gln Lys Lys Lys Pro Thr Leu Lys Arg Arg Glu Arg  
    85                                      90                                      95  
 Met Cys Met Lys Ile Glu Asn  
    100

## (2) INFORMATION FOR SEQ ID NO:116:

- (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 88 amino acids  
     (B) TYPE: amino acid  
     (C) STRANDEDNESS:  
     (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

Met Asn Tyr Ile Pro Thr Gln Thr Phe Tyr Gly Arg Arg Trp Arg Pro  
 1                      5                      10                      15  
 Arg Pro Ala Phe Arg Pro Trp Gln Val Ser Met Gln Pro Thr Pro Thr  
                          20                                      25                                      30  
 Met Val Thr Pro Met Leu Gln Ala Pro Asp Leu Gln Ala Gln Gln Met  
                          35                                      40                                      45  
 Gln Gln Leu Ile Ser Ala Val Ser Ala Leu Thr Thr Lys Gln Asn Val  
                          50                                      55                                      60



```

Met Asn Tyr Ile Pro Thr Gln Thr Phe Tyr Gly Arg Arg Trp Arg Pro
1          5          10          15
Arg Pro Ala Phe Arg Pro Trp Gln Val Ser Met Gln Pro Thr Pro Thr
          20          25          30
Met Val Thr Pro Met Leu Gln Ala Pro Asp Leu Gln Ala Gln Gln Met
          35          40          45
Gln Gln Leu Ile Ser Ala Val Ser Ala Leu Thr Thr Lys Gln Asn Val
          50          55          60
Lys Ala Pro Lys Gly Gln Arg Gln Lys Lys Gln Gln Lys Pro Lys Glu
65          70          75          80
Lys Lys Glu Asn Gln Lys Lys Lys Pro Thr Gln Lys Lys Lys Gln Gln
          85          90          95
Gln Lys Pro Lys Pro Gln Ala Lys Lys Lys Lys Pro Gly Arg Arg Glu
          100          105          110
Arg Met Cys Met Lys Ile Glu Asn
          115          120

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(2) INFORMATION FOR SEQ ID NO:115:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 103 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS:  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

Met Asn Tyr Ile Pro Thr Gln Thr Phe Tyr Gly Arg Arg Trp Arg Pro

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

TATATCTCGA GGGTGGTGTG GTAGTATTAG TCAG

34

(2) INFORMATION FOR SEQ ID NO:112:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

TATATGATAT CAAAAGCCT GAACTCACCG CGACG

35

(2) INFORMATION FOR SEQ ID NO:113:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

ATATAGGATC CTCAGTTAGC CTCCCCCATC TCCCG

35

(2) INFORMATION FOR SEQ ID NO:114:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 120 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS:

(D) TOPOLOGY: linear

CAATCTTCCT CAGGCCTTAG T

21

(2) INFORMATION FOR SEQ ID NO:109:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

TCCTAAATAG TCAGCATAGT A

21

(2) INFORMATION FOR SEQ ID NO:110:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

TCCTAAATAG TCAGCATAGT T

21

(2) INFORMATION FOR SEQ ID NO:111:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (2) INFORMATION FOR SEQ ID NO:106:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:106:

GTCOGTTTGT CGTGCAACTG A

21

## (2) INFORMATION FOR SEQ ID NO:107:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

CAATCTTCCT CACGCCTTAG C

21

## (2) INFORMATION FOR SEQ ID NO:108:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

ATCCGACCAG CAAAACTCGA TGTACTTCCG AGGAACTGAT GTGCATAATG CATCAGGCTG 11460  
 GTACATTAGA TCCCCGCTTA CCGCGGGCAA TATAGCAACA CTAAAACTC GATGTACTTC 11520  
 CGAGGAAGCG CAGTGCATAA TGCTGGGCAG TGTTGCCACA TAACCACTAT ATTAACCATT 11580  
 TATCTAGCGG ACGCCAAAAA CTCAATGTAT TTCTGAGGAA GCGTGGTGCA TAATGCCACG 11640  
 CAGCGTCTGC ATAACCTTTA TTATTCTTT TATTAATCAA CAAAATTTG TTTTAAACAT 11700  
 TTCAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 11740

(2) INFORMATION FOR SEQ ID NO:104:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

Ser Ile Leu Gly Ser Arg  
 1 5

(2) INFORMATION FOR SEQ ID NO:105:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

GTCCGTTTGT CGTGCAACTG C

CTGTTCCAAA TGTGCCACAG ATACCGTATA AGGCACTTGT TGAAAGGGCA GGGTATGCCC 10140  
CGCTCAATTT GGAGATCACT GTCATGTCCT CGGAGGTTTT GCCTTCCACC AACCAAGAGT 10200  
ACATTACCTG CAAATTCACC ACTGTGGTCC CCTCCCCAAA AATCAAATGC TGGGGCTCCT 10260  
TGGAATGTCA GCGGGCCGCT CATGCAGACT ATACCTGCAA GGTCTTGGGA GGGGTCTACC 10320  
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(2) INFORMATION FOR SEQ ID NO:103:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11740 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

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ACCACTTTCA GCAGTGOGAA GACCATGGG CGACCTTAAA AACCCCTTTCG CGTTGGGCCC 3840  
TGAATTGCCT TAACCCAGGA GGCACCCTCG TGGTGAAGTC CTATGGCTAC GCGACCGCA 3900  
ACAGTGAGGA CGTAGTCACC GCTCTTGCCA GAAAGTTTGT CAGGGTGTCT GCAGCGAGAC 3960  
CAGATTGTGT CTCAAGCAAT ACAGAAATGT ACCTGATTTT CCGACAATA GACAACAGCC 4020  
GTACACGGCA ATTCACCCCG CACCATCTGA ATTGGGTGAT TTGTCGGTG TATGAGGGTA 4080  
CAAGAGATGG AGTTGGAGCC GCGCGTCAT ACGCACCAA AAGGGAGAAT ATTGCTGACT 4140  
GTCAAGAGGA AGCAGTTGTC AACGCAGCCA ATCGCTGGG TAGACCAGGC GAAGGAGTCT 4200  
GCGTGCCAT CTATAAAGT TGGCGACCA GTTTTACCGA TTCAGCCAG GAGACAGCA 4260  
CCGCAAGAAT GACTGTGTGC CTAGGAAAGA AAGTGATCCA CGCGTGGC CCTGATTTCC 4320  
GGAAGCACC AGAAGCAGAA GCCTTGAAAT TGCTACAAA CGCCTACCAT GCAGTGGCAG 4380  
ACTTAGTAAA TGAACATAAC ATCAAGTCTG TCGCCATTCC ACTGCTATCT ACAGGCATTT 4440  
ACGCAGCCGG AAAAGACCGC CTTGAAGTAT CACTTAACTG CTTGACAACC GCGCTAGACA 4500  
GAACTGAGC GGACGTAACC ATCTATTGCC TGGATAAGAA GTGGAAGGAA AGAATCGACG 4560  
CGGCACTCCA ACTTAAGGAG TCTGTAACAG AGCTGAAGGA TGAAGATATG GAGATCGACG 4620  
ATGAGTTAGT ATGGATCCAT CCAGACAGTT GCTTGAAGGG AAGAAAGGA TTCAGTACTA 4680  
CAAAAGGAAA ATTGTATTGG TACTTGAAG GCACCAAATT CCATCAAGCA GCAAAAGACA 4740  
TGGCGGAGAT AAAGGTCTG TTCCCTAATG ACCAGGAAAG TAATGAACAA CTGTGTGCCT 4800  
ACATATTGGG TGAGACCATG GAAGCAATCC GCGAAAAGTG CCGGTGAC CATAACCGT 4860  
CGTCTAGCCC GCGAAAAG TGGCGTGCC TTGTCATGTA TGCCATGACG CCAGAAAGGG 4920  
TCCACAGACT TAGAAGCAAT AACGTCAAAG AAGTTACAGT ATGCTCCTCC ACCCCCCTTC 4980  
CTAAGCACA AATTAAGAAT GTTCAGAAG TTCAGTGAC GAAAGTAGTC CTGTTTAATC 5040

TGCTAAGACT GAGGGGTATG CAGATTACGT CGAAGACAGT AGATTGGTT ATGCTCAACG 2400  
GATGCCACAA AGCGGTAGAA GTGCTGTACG TTGACGAAGC GTTGGGTGC CAGCAGGAG 2460  
CACTACTTGC CTTGATTGCT ATCGTCAGGC CCGCAAGAA GGTAGTACTA TGGGAGACC 2520  
CCATGCAATG CGGATTCTTC AACATGATGC AACTAAAGT ACATTTCAT CACCCTGAAA 2580  
AAGACATATG CACCAAGACA TTCTACAAGT ATATCTCCG GGGTTGCACA CAGCCAGTTA 2640  
CAGCTATTGT ATCGACACTG CATTACGATG GAAAGATGAA AACCACGAAC CGTGCAAGA 2700  
AGAACATTGA AATGATATT ACAGGGGCCA CAAAGCCGAA GCCAGGGGAT ATCATCCTGA 2760  
CATGTTTCG CGGGTGGGT AAGCAATTGC AAATCGACTA TCCGGACAT GAAGTAATGA 2820  
CAGCGCGGC CTCACAAGG CTAACCAGAA AAGGAGTGTA TCCGTCCG CAAAAAGTCA 2880  
ATGAAAACCC ACTGTACCG ATCACATCAG AGCATGTGAA CGTGTGCTC ACCCGCACTG 2940  
AGGACAGGCT AGTGTGSAAG ACCTTGCAG GCGACCATG GATTAAGCAG CCCACTAACA 3000  
TACCTAAAGG AAACCTTCAG GCTACTATAG AGGACTGGGA AGCTGAACAC AAGGAATAA 3060  
TTGCTGCAAT AAACAGCCCC ACTCCCGTG CCAATCGTT CAGCTGCAAG ACCAACGTTT 3120  
GCTGGGCGAA AGCATTGGAA CGATACTAG CCACGGCGG TATCGTACTT ACCGGTTGCC 3180  
AGTGAGCGA ACTGTTCCCA CAGTTTGGG ATGACAAACC ACATTGGCC ATTTACGCT 3240  
TAGACGTAAT TTGCATTAAG TTTTTCGGCA TGGACTTGAC AAGCGGACTG TTTTCTAAAC 3300  
AGAGCATCCC ACTAAGTAC CATCCCGCG ATTGAGGAG GCGGTAGCT CATTGGGACA 3360  
ACAGCCAGG AACCGCAAG TATGGGTACG ATCAAGCCAT TCGCGCGAA CTCTCCGTA 3420  
GATTTCCGT GTTCAGCTA GCTGGGAAGG GCACACAAC TGATTTGCAG ACGGGGAGAA 3480  
CCAGAGTTAT CTCTGCACAG CATAACCTGG TCCCGGTGAA CGCAATCTT CCTACGCT 3540  
TAGTCCCGA GTACAAGGAG AAGCAACCG GCGCGTCAA AAAATTCTTG AACCAGTTCA 3600  
AACACCACTC AGTACTTGTG GTATCAGAGG AAAAAATTGA AGCTCCCGT AAGAGAATCG 3660

CCGTGGGATA CGGGTTACA CACAATAGCG AGGGCTTCTT GCTATGCAAA GTTACTGACA 1020  
CAGTAAAAGG AGAACGGGTA TCGTTCCTG TGTGCACGTA CATCCGGCC ACCATATGCG 1080  
ATCAGATGAC TGGTATAATG GCCACGGATA TATCACCTGA CGATGCACAA AACTTCTGG 1140  
TTGGGCTCAA CCAGCGAATT GTCATTAACG GTAGGACTAA CAGGAACACC AACACCATGC 1200  
AAAATTACCT TCTGCGATC ATAGCACAAG GGTTCAGCAA ATGGGCTAAG GAGCGCAAGG 1260  
ATGATCTTGA TAACGAGAAA ATGCTGGGTA CTAGAGAACG CAAGCTTACG TATGGCTGCT 1320  
TGTGGGGTT TCGCACTAAG AAAGTACATT CGTTTTATCG CCCACCTGGA ACGCAGACCT 1380  
GGTAAAAGT CCCAGCCTCT TTAGCGCTT TTCCCATGTC GTCOGTATGG ACGACCTCTT 1440  
TGCCCATGTC GCTGAGGCAG AAATTGAAAC TGGCATTGCA ACCAAAGAAG GAGGAAAAAC 1500  
TGCTGCAGGT CTOGGAGGAA TTAGTCATGG AGGCCAAGGC TGCTTTTGAG GATGCTCAGG 1560  
AGGAAGCCAG AGCGGAGAAG CTCOGAGAAG CACTTCCACC ATTAGTGGCA GACAAAGGCA 1620  
TCGAGGCAGC CGCAGAAGTT GTCTGGAAG TGGAGGGGCT CCAGGCGGAC ATOGGAGCAG 1680  
CATTAGTTGA AACCCGCGC GGTACGTAA GGATAATACC TCAAGCAAAT GACCGTATGA 1740  
TCGGACAGTA TATCGTTGTC TCGCCAACT CTGTGCTGAA GAATGCCAAA CTGCGACCAG 1800  
CGCACCGCT AGCAGATCAG GTTAAGATCA TAACACACTC CGGAAGATCA GGAAGGTACG 1860  
CGGTGGAACC ATACGACCT AAAGTACTGA TGCCAGCAGG AGGTGCGTA CCATGGCCAG 1920  
AATTCCTAGC ACTGAGTGAG AGCGCCAGT TAGTGTACAA CGAAAGAGAG TTTGTGAACC 1980  
GCAAACTATA CCACATTGCC ATGCATGGCC CGCCAAGAA TACAGAAGAG GAGCAGTACA 2040  
AGGTTACAAA GGCAGAGCTT GCAGAAACAG AGTAOGTGTT TGAGGTGGAC AAGAAGCGTT 2100  
GGTTAAGAA GGAAGAAGCC TCAGGTCTGG TCCTCTGGG AGAACTGACC AACCTCCCT 2160  
ATCATGAGCT AGCTCTGGAG GGA CTGAAGA CCGACCTGC GGTCCCGTAC AAGGTGAAA 2220  
CAATAGGAGT GATAGGCACA CCGGGTGG GCAAGTCAGC TATTATCAAG TCAACTGTCA 2280  
CGGCACGAGA TCTTGTTACC AGCGAAAGA AAGAAAATTG TCGGAAATT GAGGCGACG 2340

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8000 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

ATTGAAGGCG TAGTACACAC TATTGAATCA AACAGCCGAC CAATTGCACT ACCATCACAA	60
TGGAGAAGCC AGTAGTAAAC GTAGACGTAG ACCCCCAGAG TCGTTTGTG GTGCAACTGC	120
AAAAAAGCTT CCGCAATTT GAGGTAGTAG CACAGCAGGT CACTCCAAAT GACCATGCTA	180
ATGCCAGAGC ATTTTGGCAT CTGGCCAGTA AACTAATCGA GCTGGAGGTT CCTACCACAG	240
CGACGATCTT GGACATAGGC AGCGACCCGG CTGTTAGAAT GTTTTCCGAG CACCAGTATC	300
ATTGTGTCTG CCCCATGGGT AGTCCAGAAG ACCCGGACCG CATGATGAAA TACGCCAGTA	360
AACTGGGGGA AAAAGGGTGC AAGATTACAA ACAAGAACTT GCATGAGAAG ATTAAGGATC	420
TCGGACCGT ACTTGATACG CCGGATGCTG AAACACCATC GCTCTGCTTT CACAACGATG	480
TTACCTGCAA CATGGGTGCC GAATATTCCG TCATGCAGGA CGTGTATATC AACGCTCCCG	540
GAACTATCTA TCATCAGGCT ATGAAAGGCG TGGGACCCCT GTACTGGATT GGCTTCGACA	600
CCACCCAGTT CATGTTCTCG GCTATGGCAG GTTCGTACCC TGGTACAAC ACCAACTGGG	660
CCGACGAGAA AGTCCTTGAA GCGGTAAAC TCGGACTTTG CAGCACAAG CTGAGTGAAG	720
GTAGGACAGG AAAATTGTG ATAATGAGGA AGAAGGAGTT GAAGCCCGGG TGGGGGTTT	780
ATTCTCCGT AGGATCGACA CTTTATCCAG AACACAGAGC CAGCTTGCAG AGCTGGCATC	840
TTCCATGGT GTTCCACTTG AATGGAAAGC AGTGTACAC TTGCGCTGT GATACAGTGG	900
TGAGTTGGA AGGCTACGTA GTGAAGAAAA TCACCATCAG TCCGGGATC ACGGGAGAAA	960

CGACTGCTTA CTTATGCGGG ATTCACCGGG AATTAGTGCG TAGGCTTAAG GCCGTCTTGC 6780  
TTCCAAACAT TCACACGCTT TTTGACATGT CGGCGGAGGA TTTTGATGCA ATCATAGCAG 6840  
AACACTTCAA GCAAGGCGAC CCGGTACTGG AGACGGATAT CGCATCATTG GACAAAAGCC 6900  
AAGACGACGC TATGGCGTTA ACCGGTCTGA TGATCTTGA GGACCTGGGT GTGGATCAAC 6960  
CACTACTCGA CTTGATCGAG TGGCGCTTTG GAGAAATATC ATCCACCCAT CTACCTACGG 7020  
GTACTCGTTT TAAATTGCGG GCGATGATGA AATCCGGAAT GTTCCTCACA CTTTTTGTCA 7080  
ACACAGTTTT GAATGTGTT ATCGCCAGCA GAGTACTAGA AGAGCGGCTT AAAACGTCCA 7140  
GATGTGCAGC GTTCATTGGC GACGACAACA TCATACATGG AGTAGTATCT GACAAAGAAA 7200  
TGGCTGAGAG GTGGGCCACC TGGCTCAACA TGGAGGTAA GATCATGAC GCAGTCATCG 7260  
GTGAGAGACC ACCTTACTTC TGGCGGGAT TTATCTTGCA AGATTGGTT ACTTCCACAG 7320  
CGTGCGCGT GGCGGATCCC CTGAAAAGGC TGTTTAAGTT GGGTAAACCG CTCCCAGCCG 7380  
ACGACGAGCA AGACGAAGAC AGAAGACGG CTCTGCTAGA TGAAACAAAG GCGTGGTTTA 7440  
GAGTAGGTAT AACAGGCACT TTAGCAGTGG CCGTGAAGAC CCGGTATGAG GTAGACAATA 7500  
TTACACCTGT CCTACTGGCA TTGAGAACTT TTGCCAGAG CAAAAGAGCA TTCCAAGCCA 7560  
TCAGAGGGA AATAAAGCAT CTCTACGGTG GTCCTAAATA GTCAGCATAG TTCATTTTAT 7620  
CTGACTAATA CTACAACACC ACCACCATGA ATAGAGGATT CTTTAACATG CTCGGCGGCC 7680  
GCCCCTTCCC GGGCCCCACT GCCATGTGGA GCGCGGGAG AAGGAGGAG GCGGCCCCGA 7740  
TGCTGQCG CAACGGGCTG GCTTCTCAA TCCAGCAACT GACCACAGCC GTCAGTGCCC 7800  
TAGTCATTGG ACAGGCAACT AGACCTCAAC CCCCATGTCC ACGCCCGCCA CGGCGCCAGA 7860  
AGAAGCAGGC GCGCAAGCAA CCACCGAAGC CGAAGAAACC AAAAACGCAG GAGAAGAAGA 7920  
AGAAGCAACC TGCAAAACCC AAACCGGAA AGAGACAGCG CATGGCACTT AAGTTGGAGG 7980  
CGACAGATT GTTCGAGTC 8000

(2) INFORMATION FOR SEQ ID NO:102:

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CTTTTGGTGG GGTATCCATG TCCCTCGGAT CAATTTTCTGA CCGAGAGACG GCCCGCCAGG 5520  
CAGCGGTACA ACCCCTGGCA ACAGGCCCCA CGGATGTGCC TATGTCTTTC GGATCGTTTT 5580  
COGACGGAGA GATTGATGAG CTGAGCCGCA GAGTAACTGA GTCOGAACCC GTCCTGTTTG 5640  
GATCATTTGA ACCGGGGGAA GTGAACTCAA TTATATGTC CCGATCAGCC GTATCTTTTC 5700  
CTCTACGCAA GCAGAGAGGT AGACGCAGGA GCAGGAGGAC TGAATACTGA CTAACCGGGG 5760  
TAGGTGGGTA CATATTTTCTG ACGACACAG GCCCTGGGCA CTTGCAAAAG AAGTCGGTTC 5820  
TGCAGAACCA GCTTACAGAA CGACCTTGG AGCACAATGT CCTGGAAAGA ATTCATGCCC 5880  
CGGTGCTCGA CACGTGAAA GAGGAACAAC TCAAACCTAG GTACCAGATG ATGCCCACCG 5940  
AAGCCAACAA AAGTAGGTAC CAGTCTCGTA AAGTAGAAAA TCAGAAAGCC ATAACCACTG 6000  
AGCGACTACT GTCAGGACTA CGACTGTATA ACTCTGCCAC AGATCAGCCA GAATGCTATA 6060  
AGATCACCTA TCOGAAACCA TTGTACTCCA GTAGCGTACC GGOGAACTAC TCGATCCAC 6120  
AGTTCGCTGT AGCTGTCTGT AACAACTATC TGCATGAGAA CTATCOGACA GTAGCATCTT 6180  
ATCAGATTAC TGAOGAGTAC GATGCTTACT TGGATATGGT AGACGGGACA GTCGCCTGCC 6240  
TGGATACTGC AACCTTCTGC CCGCTAAGC TTAGAAGTTA CCGAAAAAA CATGAGTATA 6300  
GAGCCCOGAA TATCOGCAGT GCGGTTCCAT CAGCGATGCA GAACACGCTA CAAAATGTGC 6360  
TCATTGCCGC AACTAAAAGA AATTGCAACG TCAOGCAGAT GCGTGAAGT CCAACACTGG 6420  
ACTCAGGAC ATTCAATGTC GAATGCTTTC GAAAATATGC ATGTAATGAC GAGTATTGGG 6480  
AGGAGTTGCG TCGGAAGCCA ATTAGGATTA CCACTGAGTT TGTACCGCA TATGTAGCTA 6540  
GACTGAAAGG CCCTAAGGCC GCOGCACTAT TTGCAAAGAC GTATAATTTG GTCCCATTCG 6600  
AAGAAGTGCC TATGGATAGA TTGTCATGG ACATGAAAAG AGACGTGAAA GTTACACCAG 6660  
GCAOGAAACA CACAGAAGAA AGACCGAAAG TACAAGTGAT ACAAGCCGCA GAACCCCTGG 6720

GTACACGGCA ATTCACCCCG CACCATCTGA ATTGCGTGAT TTGTCCTGTG TATGAGGGTA 4080  
CAAGAGATGG AGTTGGAGCC GCGCGTCAT ACCGCACCA AAGGGAGAAT ATTGCTGACT 4140  
GTCAAGAGGA AGCAGTTGTC AACGCAGCCA ATCCGCTGGG TAGACCAGGC GAAGGAGTCT 4200  
GCGTGCCAT CTATAAAGT TGGCCGACCA GTTTTACCGA TTCAGCCACG GAGACAGGCA 4260  
CCGCAAGAAT GACTGTGTGC CTAGGAAAGA AAGTGATCCA CCGGTCGGC CCTGATTTCC 4320  
GGAAGCACCC AGAAGCAGTA GCCTTGAAAT TGCTACAAA CCGCTACCAT GCAGTGGCAG 4380  
ACTTAGTAAA TGAACATAAC ATCAAGTCTG TCGCCATTCC ACTGCTATCT ACAGGCATTT 4440  
ACGCAGCCGG AAAAGACCGC CTTGAAGTAT CACTTAACTG CTTGACAACC GCGCTAGACA 4500  
GAACTGACGC GGAAGTAACC ATCTATTGCC TGGATAAGAA GTGGAAGGAA AGAATCGACG 4560  
CGGCACTCCA ACTTAAGGAG TCTGTAACAG AGCTGAAGGA TGAAGATATG GAGATCGACG 4620  
ATGAGTTAGT ATGGATCCAT CCAGACAGTT GCTTGAAGGG AAGAAAGGGA TTCAGTACTA 4680  
CAAAAGGAAA ATTGTATTGG TACTTCGAAG GCACCAAATT CCATCAAGCA GCAAAAGACA 4740  
TGGCGGAGAT AAAGGTCTG TTCCCTAATG ACCAGGAAAG TAATGAACAA CTGTGTGCCT 4800  
ACATATTGGG TGAGACCATG GAAGCAATCC GCGAAAAGTG CCGGTCGAC CATAACCGT 4860  
CGTTTAGCCC GCCAAAACG TTGCGTGCC TTTGCATGTA TGCCATGACG CCAGAAAGGG 4920  
TCCACAGACT TAGAAGCAAT AACGTCAAAG AAGTTACAGT ATGCTCCTCC ACCCCCCTTC 4980  
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CGCACACTCC CGCATTGGTT CCGCCCGTA AGTACATAGA AGTGCCAGAA CAGCCTACCG 5100  
CTCCTCCTGC ACAGGCCGAG GAGGCCCGG AAGTTGTAGC GACACGTCA CCATCTACAG 5160  
CTGATAACAC CTGCTTGAT GTCACAGACA TCTCACTGGA TATGGATGAC AGTAGCGAAG 5220  
GCTCACTTTT TTGAGCTTT AGCGATCGG ACAACTCTAT TACTAGTATG GACAGTTGGT 5280  
CGTCAGGACC TAGTTCATA GAGATAGTAG ACCGAAGGCA GGTGGTGGTG GCTGACGTTT 5340  
ATGCGGTCCA AGAGCCTGCC CCTATTCCAC CGCCAAGGCT AAAGAAGATG GCCCGCCTGG 5400

AGAACATTGA AATCGATATT ACAGGGGCCA CAAAGCCGAA GCCAGGGGAT ATCATCCTGA 2760  
CATGTTTCCG CCGGTGGGT AAGCAATTGC AAATCGACTA TCCCGGACAT GAAGTAATGA 2820  
CAGCCGGGC CTCACAAGGG CTAACCAGAA AAGGAGTGTA TGGCGTCGG CAAAAAGTCA 2880  
ATGAAAACCC ACTGTAACGG ATCACATCAG AGCATGTGAA CGTGTGCTC ACCCGCACTG 2940  
AGGACAGGCT AGTGTGAAA ACCTTGACGG GCGACCCATG GATTAAGCAG CTCCTAACA 3000  
TACCTAAAGG AACTTTTCAG GCTACTATAG AGGACTGGGA AGCTGAACAC AAGGAATAA 3060  
TTGCTGCAAT AACAGCCCC ACTCCCGTG CCAATCGTT CAGCTGCAAG ACCAAGTTT 3120  
GCTGGGCGAA AGCATTGGAA CCGATACTAG CCACGGCCGG TATCGTACTT ACCGGTTGCC 3180  
AGTGGAGCGA ACTGTTCCA CAGTTTGCG ATGACAAACC ACATTGGCC ATTTACGCCT 3240  
TAGACGTAAT TTGCATTAAG TTTTTCGGCA TGGACTTGAC AAGCGGACTG TTTTCTAAAC 3300  
AGAGCATCCC ACTAAGTAC CATCCCGCG ATTCAGGAG GCGGTAGCT CATTGGGACA 3360  
ACAGCCCAGG AACCGCAAG TATGGGTAC ATCAGCCAT TGGCGCGAA CTCTCCGTA 3420  
GATTTCCGT GTTCCAGCTA GCTGGGAAGG GCACACAACT TGATTTGAG ACGGGGAGAA 3480  
CCAGAGTTAT CTCTGCACAG CATAACCTGG TCCCGGTGAA CGCAATCTT CCTCAGCCT 3540  
TAGTCCCGA GTACAAGGAG AAGCAACCG GCCCGGTGAA AAAATTCTTG AACCAGTTCA 3600  
AACACCACTC AGTACTTGTG GTATCAGAGG AAAAAATTGA AGCTCCCGT AAGAGAATCG 3660  
AATGGATGCG CCGATTGGC ATAGCGGTG CAGATAAGAA CTACAACCTG GCTTTGGGT 3720  
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ACAGTGAGGA CGTAGTCACC GCTCTTGCCA GAAAGTTTGT CAGGGTGTCT GCAGCGAGAC 3960  
CAGATTGTGT CTCAAGCAAT ACAGAAATGT ACCTGATTTT CCGACAATA GACAACAGCC 4020



TGTGGGCGTT TCGCACTAAG AAAGTACATT CGTTTTATCG CCCACCTGGA ACGCAGACCT 1380  
GGGTAAAAGT CCCAGCCTCT TTAGCGCTT TTCCCATGTC GTCOGTATGG ACGACCTCTT 1440  
TGCCCATGTC GCTGAGGCAG AAATTGAAAC TGGCATTGCA ACCAAAGAAG GAGGAAAAAC 1500  
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AGGAAGCCAG AGGGGAGAAG CTCGAGAAG CACTTCCACC ATTAGTGGCA GACAAAGGCA 1620  
TCGAGGCAGC CGCAGAAGTT GTCTGCGAAG TGGAGGGGCT CCAGGCGGAC ATCGGAGCAG 1680  
CATTAGTTGA AACCCCGGCG GGTCACGTAA GGATAATACC TCAAGCAAAT GACCGTATGA 1740  
TCGGACAGTA TATCGTTGTC TGCCAAACT CTGTACTGAA GAATGCCAAA CTCGCACCAG 1800  
CGCACCOCCT AGCAGATCAG GTTAAGATCA TAACACACTC CGGAAGATCA GGAAGGTACG 1860  
CGGTGGAACC ATACGACGCT AAAGTACTGA TGCCAGCAGG AGGTGCCGTA CCATGGCCAG 1920  
AATTCCTAGC ACTGAGTGAG AGCGCCACGT TAGTGACAA CGAAAGAGAG CTTGTGAACC 1980  
GCAAACTATA CCACATTGCC ATGCATGGCC CCGCCAAGAA TACAGAAGAG GAGCAGTACA 2040  
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ATCATGAGCT AGCTCTGGAG GGAAGTGAAGA CCGACCTGC GGTCCCGTAC AAGGTGAAA 2220  
CAATAGGAGT GATAGGCACA CCGGGGTGG GCAAGTCAGC TATTATCAAG TCAACTGTCA 2280  
CGGCAOGAGA TCTTGTTACC AGCGGAAAGA AAGAAAATTG TCGGAAATT GAGGCOGACG 2340  
TGCTAAGACT GAGGGGTATG CAGATTACGT CGAAGACAGT AGATTGGTT ATGCTCAACG 2400  
GATGCCACAA AGCOGTAGAA GTGCTGTACG TTGACGAAGC GTTCGGGTGC CAGCAGGAG 2460  
CACTACTTGC CTTGATTGCT ATGTCAGGC CCGCAAGAA GGTAGTACTA TGCGGAGACC 2520  
CCATGCAATG CGGATTCTTC AACATGATGC AACTAAAGGT ACATTTCAAT CACCCTGAAA 2580  
AAGACATATG CACCAAGACA TTCTACAAGT ATATCTCCCG GCGTTGCACA CAGCCAGTTA 2640  
CAGCTATTGT ATGACACTG CATTACGATG GAAAGATGAA AACCACGAAC CGTGCAAGA 2700

ATTGACGGCG TAGTACACAC TATTGAATCA AACAGCCGAC CAATGCACT ACCATCACAA	60
TGGAGAAGCC AGTAGTAAAC GTAGACGTAG ACCCCCAGAG TCGTTTGTG GTGCAACTGA	120
AAAAAAGCTT CCGCAATTT GAGGTAGTAG CACAGCAGGT CACTCCAAAT GACCATGCTA	180
ATGCCAGAGC ATTTTGGCAT CTGGCCAGTA AACTAATCGA GCTGGAGGTT CCTACCACAG	240
CGACGATCTT GGACATAGGC AGGGCACC GG CTGTTAGAAT GTTTTCCGAG CACCAGTATC	300
ATTGTGTCTG CCCCATGCGT AGTCCAGAAG ACCCGGACCG CATGATGAAA TACGCCAGTA	360
AACTGGCGGA AAAAGCGTGC AAGATTACAA ACAAGAACTT GCATGAGAAG ATTAAGGATC	420
TCGGGACCGT ACTTGATACG CCGGATGCTG AAACACCATC GCTCTGCTTT CACAAAGATG	480
TTACCTGCAA CATGCGTGCC GAATATTCCG TCATGCAGGA CGTGTATATC AACGCTCCCG	540
GAACTATCTA TCATCAGGCT ATGAAAGGCG TGGGACCCT GTACTGGATT GGCTTCGACA	600
CCACCCAGTT CATGTTCTCG GCTATGGCAG GTTCGTACCC TGGTACAAC ACCAACTGGG	660
CGACGAGAA AGTCCTTGAA GGGGTAAACA TCGGACTTTG CAGCACAAAG CTGAGTGAAG	720
GTAGGACAGG AAAATTGTG ATAATGAGGA AGAAGGAGTT GAAGCCCGGG TGGGGGTTT	780
ATTTCTCGT AGGATCGACA CTTTATCCAG AACACAGAGC CAGCTTGCAG AGCTGGCATC	840
TTCCATCGGT GTTCCACTTG AATGGAAAGC AGTCGTACAC TTGCGCTGT GATACAGTGG	900
TGAGTTGCGA AGGCTACGTA GTGAAGAAAA TCACCATCAG TCCCGGGATC ACGGGAGAAA	960
CGTGGGATA CGGGTTACA CACAATAGCG AGGGCTTCTT GCTATGCAA GTTACTGACA	1020
CAGTAAAGG AGAACGGTA TCGTTCCCTG TGTGCAAGTA CATCCCGGCC ACCATATGGG	1080
ATCAGATGAC TGGTATAATG GCCACGGATA TATCACCTGA CGATGCACAA AAATTCTGG	1140
TTGGGCTCAA CCAGCGAATT GTCATTAAG GTAGGACTAA CAGGAACACC AACACCATGC	1200
AAAATTACCT TCTGCCGATC ATAGCACAAG GGTTCAGCAA ATGGGCTAAG GAGCGCAAGG	1260
ATGATCTTGA TAACGAGAAA ATGCTGGGTA CTAGAGAAG CAAGCTTACG TATGGCTGCT	1320

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

CGCGCCGAAT TGTGAGCGCT CACAATTCGG

30

(2) INFORMATION FOR SEQ ID NO:100:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

Pro Lys Lys Lys Lys Arg Lys  
1 5

(2) INFORMATION FOR SEQ ID NO:101:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8000 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GCCCTTTCTC TTCTTTTTTG GCTGCCCGCT TTCCAGTOGG GAAAC

45

(2) INFORMATION FOR SEQ ID NO:97:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

CCAAAAAAGA AGAGAAAGGG CGGTGGTGCT TTGTCTCCT

39

(2) INFORMATION FOR SEQ ID NO:98:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

ATATAGAGCT CTAAACTGA TGATTGATT TCAAATGC

38

(2) INFORMATION FOR SEQ ID NO:99:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

ATATAGAGCT CAGGCGTTGA AAAGATTAGC GACCG

35

(2) INFORMATION FOR SEQ ID NO:94:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TATATATTAA TTAAATAGAA TGACACCTAC TCAGACAATG CGATGC

46

(2) INFORMATION FOR SEQ ID NO:95:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

ATATACTGA GTAGCAATGG TGAAACCAGT AACGTTATAC

40

(2) INFORMATION FOR SEQ ID NO:96:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

TAGTGAACCG TATTGACGGC GTAGTACACA CTATT

35

(2) INFORMATION FOR SEQ ID NO:91:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

CGTTGAGCAT AACCGAATCT AC

22

(2) INFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

ATATAGAGCT CTTAATTAAT CTTTGTGAAG GAACCTTACT TC

42

(2) INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

TATATATATG CGGCGCTCA ATTATGTTTC TGGTTGGT

38

(2) INFORMATION FOR SEQ ID NO:88:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 42 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

TATATAGATC TGGCGCGCCT TTACCACTCC TATCAGTGAT AG

42

(2) INFORMATION FOR SEQ ID NO:89:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

TACGCGGTCA ATACGGTTCA CTAAACGAGC TCTGC

35

(2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (2) INFORMATION FOR SEQ ID NO:85:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

CACGGTCCTG AGGTGC

16

## (2) INFORMATION FOR SEQ ID NO:86:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

TATATATATC TOGAGACCGC CAAGATGTTC CGTTCCAGC CA

42

## (2) INFORMATION FOR SEQ ID NO:87:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:



- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

CCACAAGCTT GGACCACCGT AGAG

24

(2) INFORMATION FOR SEQ ID NO:83:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CCGCGTGGCG GATCCCCTG

19

(2) INFORMATION FOR SEQ ID NO:84:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

CCACGGATCC GGAAGGGACA GAAG

24

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CCACAAGCTT CCGGAGTTAC CCGAGTGACC

30

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

CCACCTTAAG CGTCGGCTTT TTCTTC

26

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

CCACCTTAAG AGAAGAGAAA GAATG

25

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

CCACGGATCC CGGCGTTCCG TCC

23

(2) INFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

CCACAAGCTT GTGCACTGGG ATCTG

25

(2) INFORMATION FOR SEQ ID NO:78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

CCACGGATCC GTGCACATGA AGTCC

25

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

ACACATTAAT TAAGTATTGG CCCCAATGGG GTCT

34

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

CCACGAATTC GGTCTAAAT AGATGC

26

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

CCACAAGCTT CCGGGGAGG CGCC

25

(2) INFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

ATATAGGCGG CCGCTCATCT TCGTGTGCTA GTCAGCATC

39

## (2) INFORMATION FOR SEQ ID NO:72:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

ACACATTAAT TAAGGATGCC GCCGGAAGCG AGAA

34

## (2) INFORMATION FOR SEQ ID NO:73:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 34 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

ATATACTCGA GACCACCACC ATGAATAGAG GATTC

35

## (2) INFORMATION FOR SEQ ID NO:69:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TATATGCGGC CGCTATTACC ACTCTTCTGT CCCTTCGGG GT

42

## (2) INFORMATION FOR SEQ ID NO:70:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

ATATACTCGA GAGCAATGTC CGCAGCACCA CTGGTCACGG CA

42

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

CATGCGAAAC GATCCTCATC CTTACAATCG TGGTTTTCAG AGG

43

(2) INFORMATION FOR SEQ ID NO:66:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

GATGAGGATC GTTTCGCATG ATTGA

25

(2) INFORMATION FOR SEQ ID NO:67:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATATATGCA TTCAGAAGAA CTCGTCAAGA AGGCGA

36

(2) INFORMATION FOR SEQ ID NO:68:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

GATATAGTGG TGAGTATCCC CG

22

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATATGGATC COGGAGATCC TTAATCTTCT CATG

34

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

TATATATGCA TCCCCCCCCC CCCCCAAG

28

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs
- (B) TYPE: nucleic acid



(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TATATGAATT CGTTTGGACA AACCACAACT AGAATG

36

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

TATATAGATC TAGTCTTATG CAATACTCTT GTAGT

35

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GGGATACTCA CCACTATATC TCGACGGTAT CGAGGTAGGG CACT

44

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

ATATATCCGG AGTCCGGCCG CTGGGGTGA GAGGCTA

37

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

ATATAGGATC CTCAGAAGAA CTGGTCAAGA AGGCGA

36

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 44 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

TATATATGAG CTCTTACAAA TAAAGCAATA GCATCACAAA TTTC

44

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 55 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

CTGTTTAAAC AGATCTTATC TCGAGTATGC GGCCGCTATG AATTCGTTTA AACGA

55

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 55 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

TCGTTTAAAC GAATTCATAG CGGCCGCATA CTOGAGATAA GATCTGTTTA AACAG

55

(2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATATAGGAT CCTAGGTTAG GTTGAATCT AAAATACACA AAC

43

(2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 12 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TCGATCCTAG GA

12

(2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 16 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GCTCTTAATT AAGAGC

16

(2) INFORMATION FOR SEQ ID NO:54:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CCTATGCGGC CGCTAGACTG GACAGCCAAT GACG

34

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CCTATTGGCC AGCCAAGACA TCATCGGGC AG

32

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TATATATCG GAAAGCTCTA AGGTAAATAT AAAATTTT

39

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43 base pairs

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

GGTAACAAGA TCTOGTGCCG TG

22

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CCTATGCGGC CGCGTGAAC CTTTGTGGCT CCTC

34

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

CCTATTGGCC AGCAGACCAA TTTATGCCTA C

31

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

COGTCAATAC GGTTCATAA ACGAGCTCTG CTTATATAGA CC

42

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCTCGTTTAG TGAACCGTAT TGACGGGTA GTACACAC

38

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CTGGCAACCG GTAAGTACGA TAC

23

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TATATGAGCT CCTCCCTTAG CCATCOGAGT GGACGTGGT CCTCCTTCGG ATGCCCAGGT 60  
CGGACCGCG 69

## (2) INFORMATION FOR SEQ ID NO:41:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TATATATAGA TCTTTGACAT TGATTATTGA CTAG 34

## (2) INFORMATION FOR SEQ ID NO:42:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:



- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TATATATGAG CTCTAATAAA ATGAGGAAAT TGCATCGCAT TGTC

44

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 43 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TATATGAATT CATAGAATGA CACCTACTCA GACAATGCGA TGC

43

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 56 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AAAAAAAAA GGGTCGGCAT GGCATCTCCA CCTCCTCGCG GTCCGACCTG GGCATC

56

(2) INFORMATION FOR SEQ ID NO:40:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AGGATCCTAG T

11

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CTCGATCCTA GGATCGAGGC

20

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GAGCTAGGAT CCTAGCTCCG

20

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

(B) TYPE: nucleic acid

TATATATATA TGCGGCGGCC GCTACGCCCC AATGATCCGA G

41

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 65 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTATAGAGCT CGTTTAACT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTG

60

AAATG

65

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TOGATCCTAG GA

12

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GAAGTGCCAG AACAGCCTAC CG

22

## (2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TATATCTCGA GGGTGGTGTT GTAGTATTAG TCAG

34

## (2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 41 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ATATACTCGA GACCATGATT GAACAAGATG GATTG

35

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TATATAGCGG CGCTCAGAA GAACTCGTCA AGAAG

35

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTGTAGATGG TGACGGTGTC G

21

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TATATCTAG ATTTTTTTT TTTTTTTT TTTTTTTT TTTTGGAA TG

52

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATATATCTAG AGCCATGGGC CACACAGGA GGCAG

35

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATATAGGATC CCTGTTATAC AGGGCGTACA CTTTC

35

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGGGTCTTCA ACTCACCGGA C

21

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAATTGACG TAGGCCTCAC TC

22

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAGTGAGGCG TAGTGAAT TG

22

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCTTTTCAGG GGATCCGCCA C

21

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTGGCGGATC CCCTGAAAAG G

21

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGGGCGGTGT GGTCGTCATG

20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear



## FURTHER INFORMATION C NTINUED FROM PCT/ISA/ 210

## 1. Claims 1-9, 18-25 and partially 17 :

An isolated nucleic acid molecule comprising an altered alphavirus protein gene which, when operably incorporated into a recombinant alphavirus particle ,

. increases the time required to reach 50% inhibition of host-cell directed macromolecular synthesis following expression in mammalian cells, or

. has a reduced level of vector-specific RNA synthesis and the same or greater level of proteins encoded by RNA transcribed from the viral junction promoter, as compared to a wild-type alphavirus particule;

an alphavirus vector construct, RNA replicon or eukaryotic layered initiation system including it; an alphavirus producer cell line comprising one of these vectors, a recombinant alphavirus particle comprising the replicon, a host cell infected by this particle or containing the replicon, pharmaceutical compositions comprising these replicon or particle and methods for utilizing these alphavirus-based vectors.

## 2. Claims 10-14 :

A Togavirus capsid particle which contains substantially no genomic or RNA vector replicon nucleic acids and pharmaceutical composition comprising thereof.

## 3. Claims 15, 16 and partially 17 :

An alphavirus structural protein expression cassette, comprising a 5' promoter which initiates synthesis of RNA from DNA, a nucleic acid molecule which encodes one or more functional alphavirus structural proteins, a selectable marker operably linked to transcription of the expression cassette , and a 3' sequence which controls transcription termination; alphavirus packaging and producer cell lines comprising cells containing this cassette.

REMARK: As far as claim 21 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 97/06010

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
See annex.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See annex

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-9, 18-25 and partially 17.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/06010

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF VIROLOGY, vol. 70, no. 1, January 1996, pages 508-519, XP002039561 DUBENSKY, T.W. ET AL.: "Sindbis virus DNA-based expression vectors: utility for in vitro and in vivo gene transfer" cited in the application see the whole document ---	1-5,17, 21-25
A	ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 772, 1 January 1995, pages 261-264, XP000600126 DRIVER D A ET AL: "LAYERED AMPLIFICATION OF GENE EXPRESSION WITH A DNA GENE DELIVERY SYSTEM" see the whole document ---	18-24
A	JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 0, no. 21A, 1995, page 406 XP002039562 DUBENSKY, T. ET AL.: "Polynucleotide based gene transfer vector derived from an alphavirus" see abstract C6-413 -----	18-24

## INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 97/06010

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C07K14/18 C12N7/04 C12N7/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols):

IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO JOURNAL, vol. 13, no. 12, 1994, EYNSHAM, OXFORD GB, pages 2925-2934, XP002039559 LEMM. J.A. ET AL.: "Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis" see the whole document ---	2
X	VIROLOGY, vol. 191, 1992, ORLANDO US, pages 932-940, XP002039560 STRAUSS, G.E. ET AL.: "Identification of the active site residues in the nsP2 proteinase of Sindbis Virus" see the whole document --- -/-	1,2

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*B\* document member of the same patent family

Date of the actual completion of the international search

3 September 1997

Date of mailing of the international search report

15-12-1997

Name and mailing address of the ISA

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Fax: (+31-70) 340-3016

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CHAMBONNET, F

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EE	Estonia						

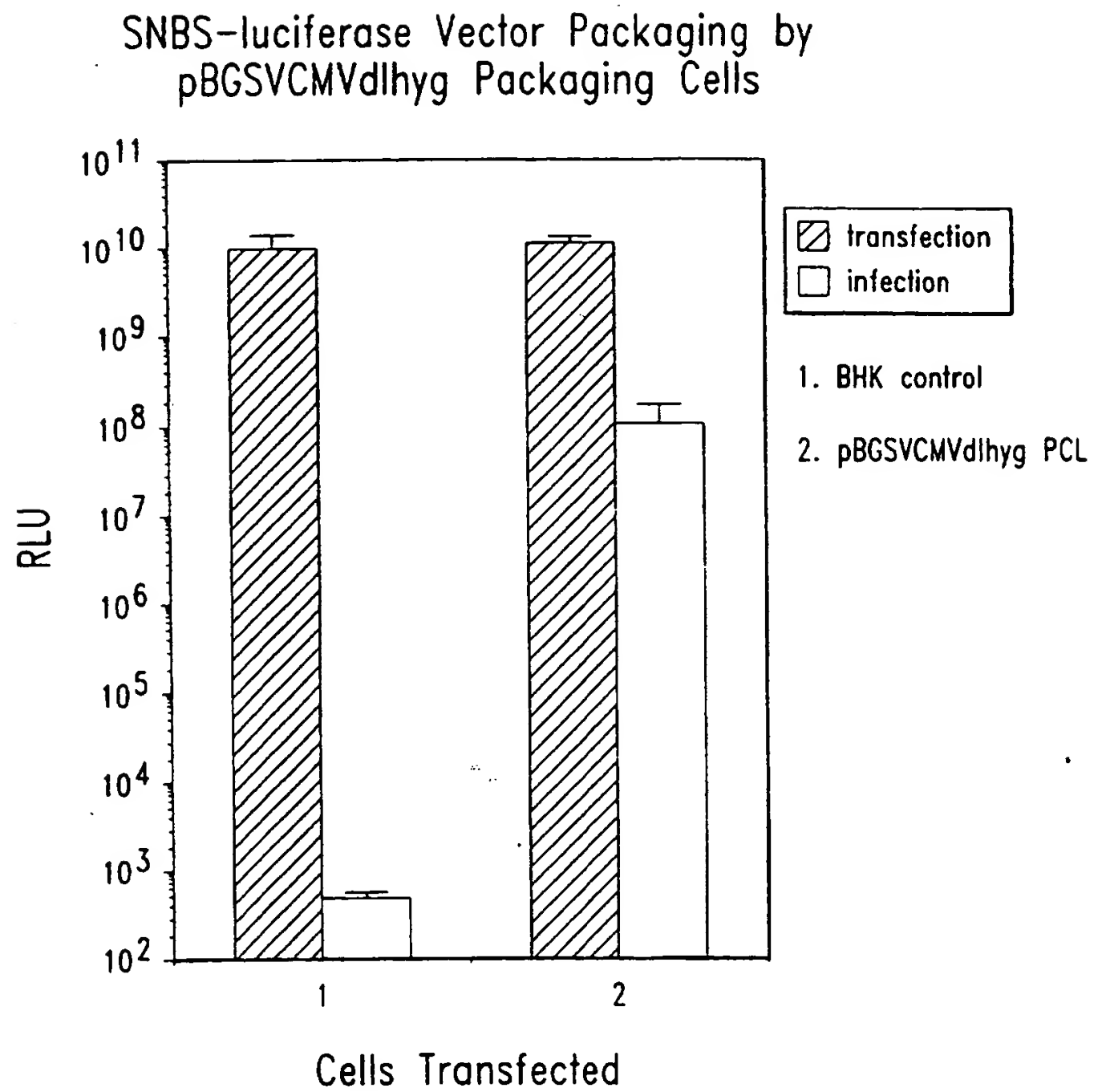


## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C12N 15/86, C07K 14/18, C12N 7/04, 7/00</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 97/38087</b> <b>(43) International Publication Date:</b> 16 October 1997 (16.10.97)
<b>(21) International Application Number:</b> PCT/US97/06010 <b>(22) International Filing Date:</b> 4 April 1997 (04.04.97)  <b>(30) Priority Data:</b> 08/628,594           5 April 1996 (05.04.96)           US 08/668,953           24 June 1996 (24.06.96)           US 08/679,640           12 July 1996 (12.07.96)           US  <b>(71) Applicants (for all designated States except US):</b> CHIRON VIAGENE, INC. [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US). WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> DUBENSKY, Thomas, W., Jr. [US/US]; P.O. Box 675205, Rancho Santa Fe, CA 92067 (US). POLO, John, M. [US/US]; 221 Witham Road, Encinitas, CA 92024 (US). BELLI, Barbara, A. [US/US]; 5850 Despejo Place, San Diego, CA 92124 (US). SCHLESINGER, Sondra [US/US]; 6320 McPherson Street, St. Louis, MO 63105 (US). DRYGA, Sergey A. [RU/US]; 6334 Southwood Avenue #3, St. Louis, MO 63105 (US). FROLOV, Ilya [RU/US]; 7515 Cromwell Drive #2SE, St. Louis, MO 63105 (US).		<b>(74) Agents:</b> McMASTERS, David, D. et al.; Seed and Berry L.L.P., 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 12 March 1998 (12.03.98)
<b>(54) Title:</b> ALPHAVIRAL VECTOR WITH REDUCED INHIBITION OF CELLULAR MACROMOLECULAR SYNTHESIS  <b>(57) Abstract</b>  <p>Isolated nucleic acid molecules are disclosed, comprising an alphavirus nonstructural protein gene which, when operably incorporated into a recombinant alphavirus particle, eukaryotic layered vector initiation system, or RNA vector replicon, has a reduced level of vector-specific RNA synthesis, as compared to wild-type, and the same or greater level of proteins encoded by RNA transcribed from the viral junction region promoter, as compared to a wild-type recombinant alphavirus particle. Also disclosed are RNA vector replicons, alphavirus vector constructs, and eukaryotic layered vector initiation systems which contain the above-identified nucleic acid molecules.</p>		



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*Fig. 39*



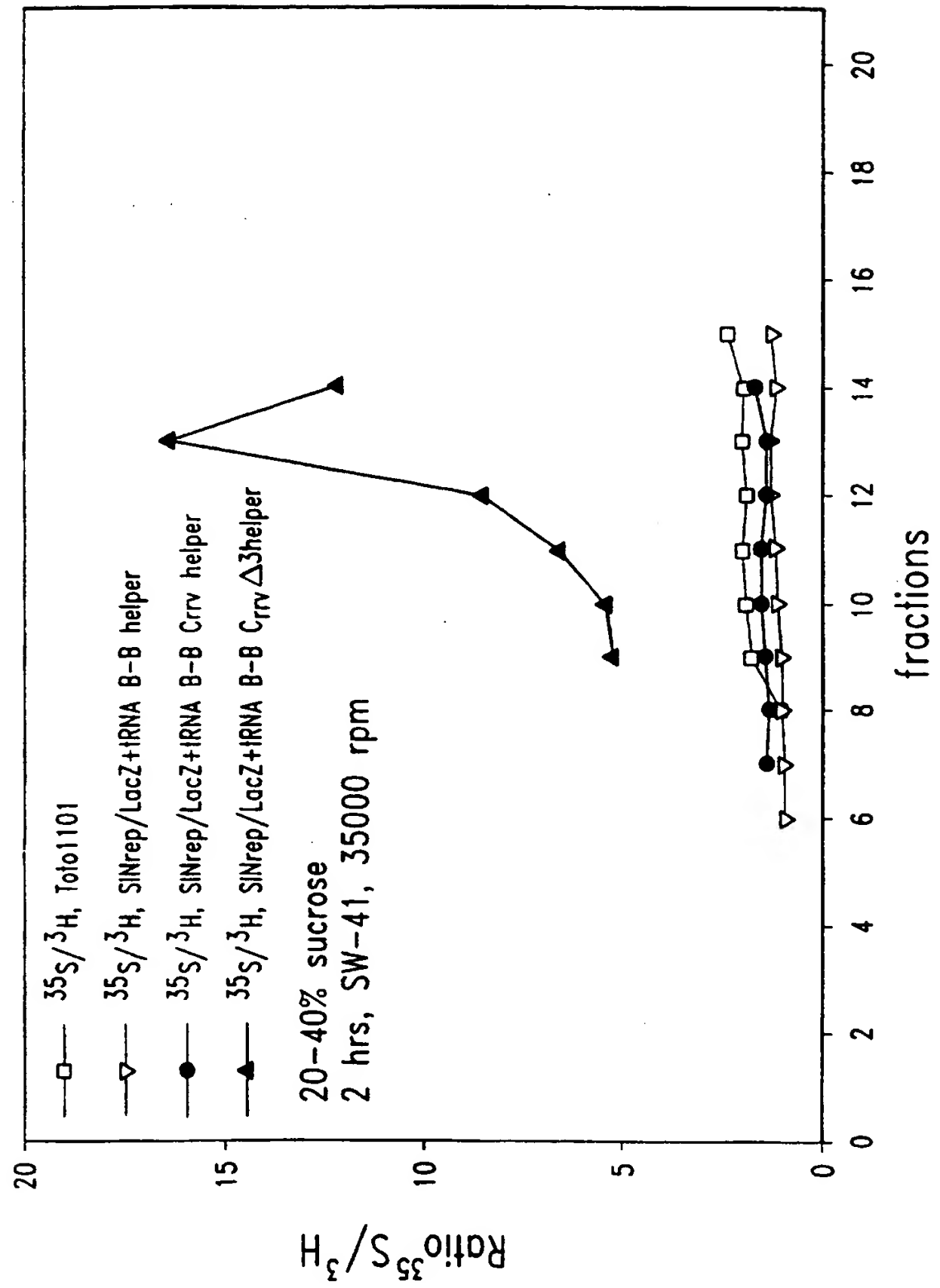


Fig. 38

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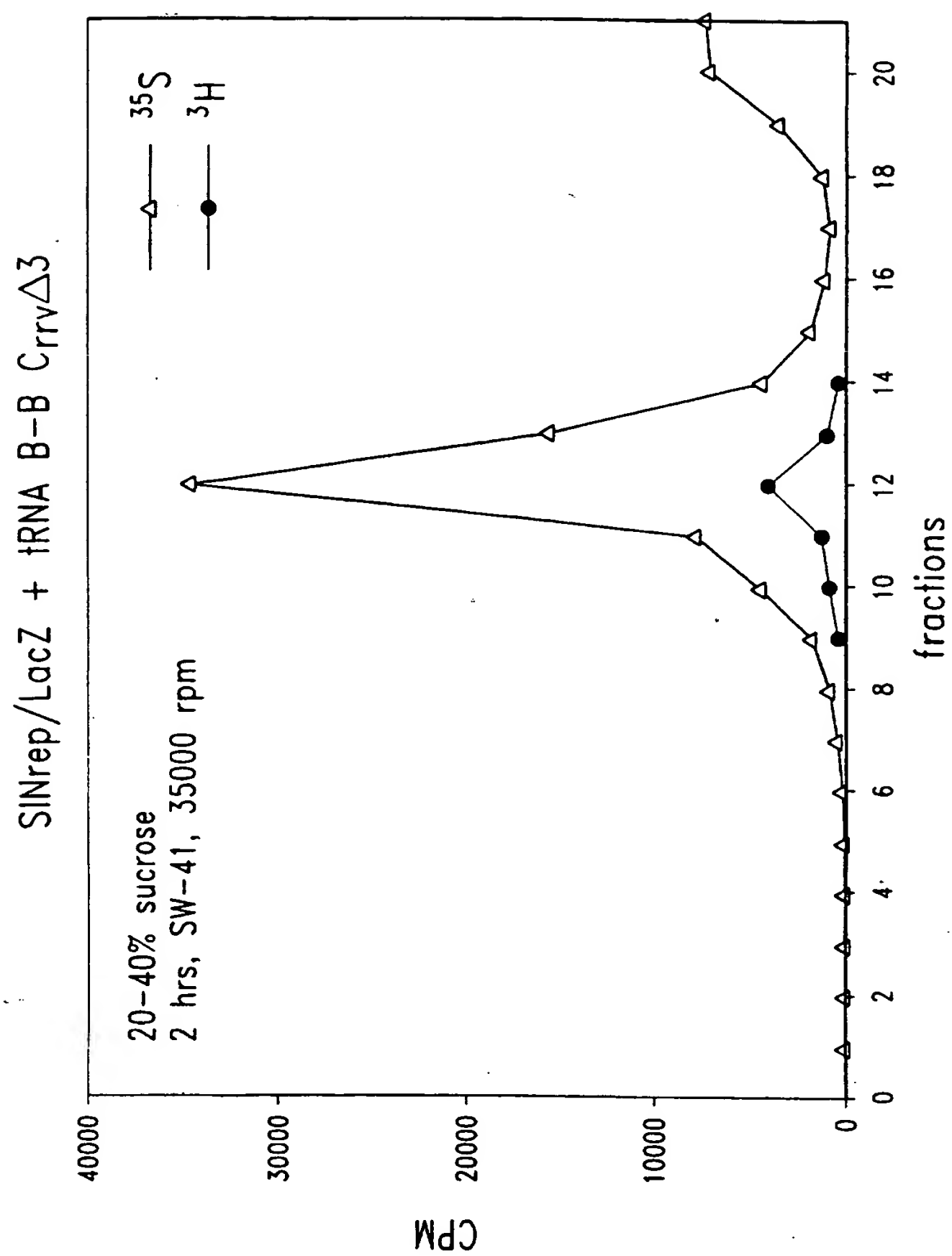


Fig. 37

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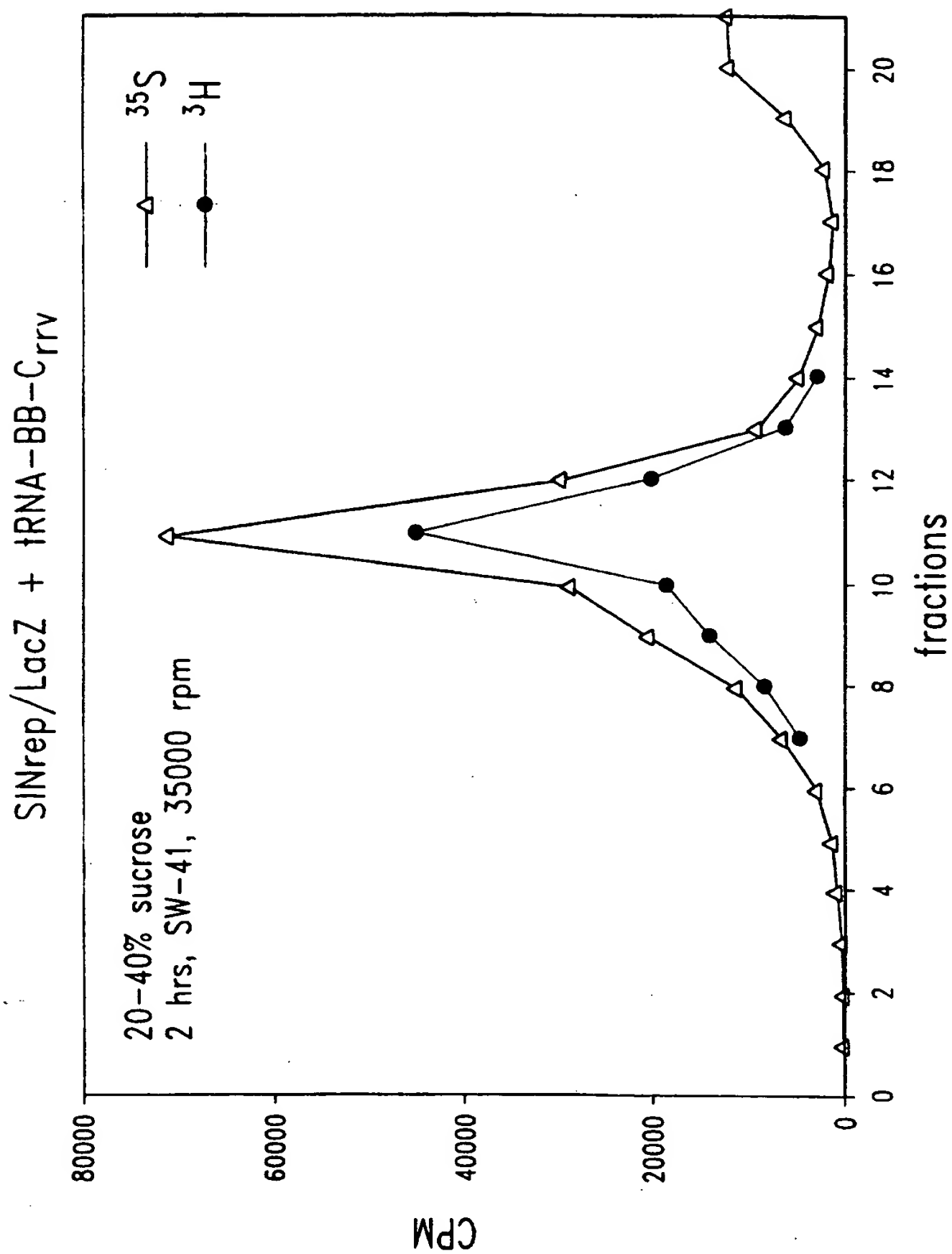


Fig. 36

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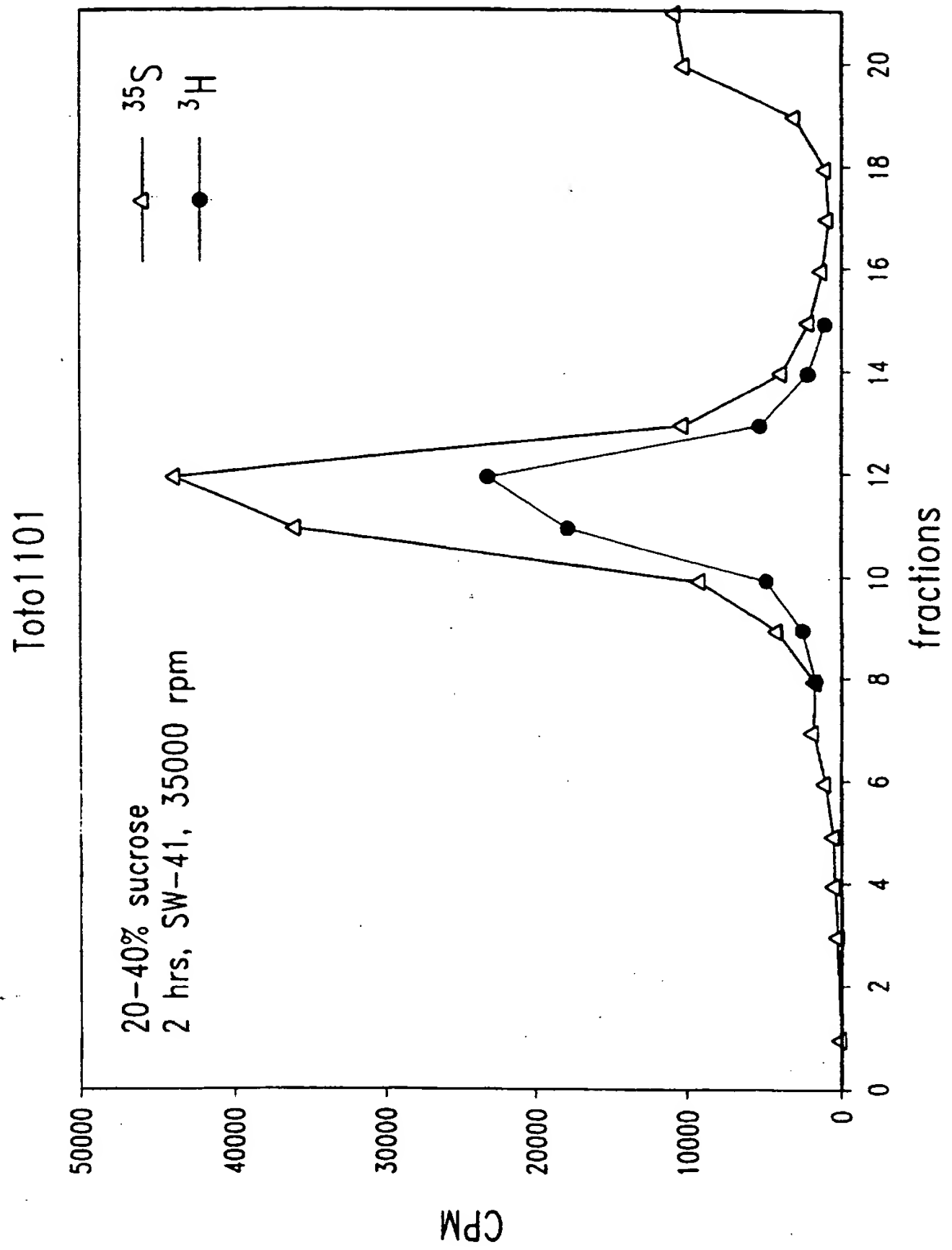


Fig. 35

	++ + + + +
Crrv	MNYIPTQTFYGRRWPRPFRPWQVSMQPTPTMVT PMLQAPDLQAQQMQQLISAVSALIT 60
CrrvΔ1	MNYIPTQTFYGRRWPRPFRPWQVSMQPTPTMVT PMLQAPDLQAQQMQQLISAVSALIT
CrrvΔ2	MNYIPTQTFYGRRWPRPFRPWQVSMQPTPTMVT PMLQAPDLQAQQMQQLISAVSALIT
CrrvΔ3	MNYIPTQTFYGRRWPRPFRPWQVSMQPTPTMVT PMLQAPDLQAQQMQQLISAVSALIT

	+ + + + + + + + + + + + + + + + +
Crrv	KQNVKAPKGQRQKKQKPKKEKKENQKKKPTQKKKQKQKPKPQAKKKKPGRRERMCMK IEN 120
CrrvΔ1	KQNVKAPKGQRQKKQKPKKEKKENQKKKPTLK-----RRERMCMK IEN
CrrvΔ2	KQNVKAPKGQRQKKQK-----RRERMCMK IEN
CrrvΔ3	KQNLK-----RRERMCMK IEN

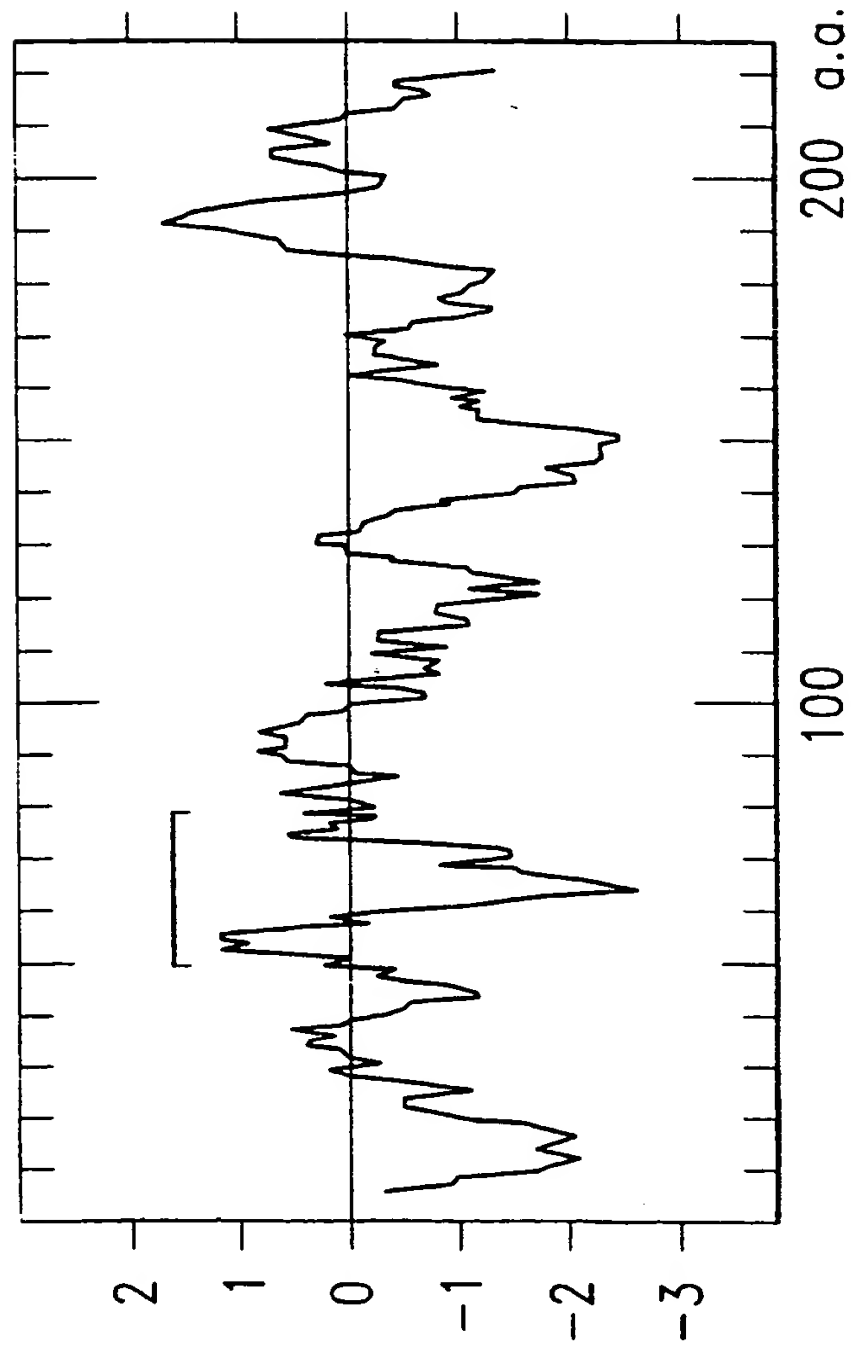
CΔ1rrv - del 8 Lys  
CΔ2rrv - del 15 Lys  
CΔ3rrv - del 19 Lys

Fig. 34

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Hydrophobicity profiles of Capsides (Kyte - Doolittle)

CΔ3rrv

*Fig. 33D*

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Hydrophobicity profiles of Capsides (Kyte - Doolittle)

CΔ2rrv

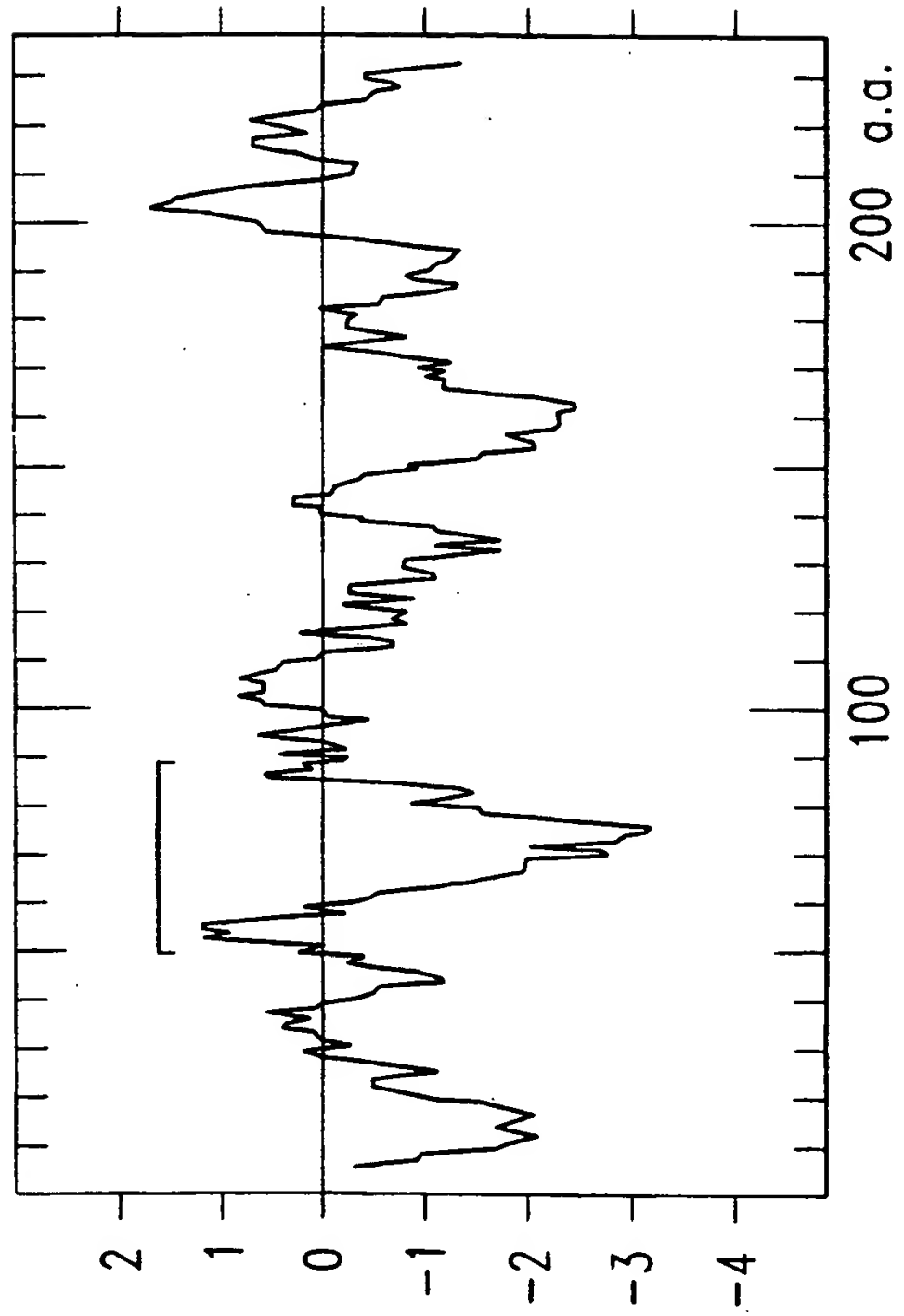


Fig. 33C

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Hydrophobicity profiles of Capsides (Kyte - Doolittle)

CΔ1rrv

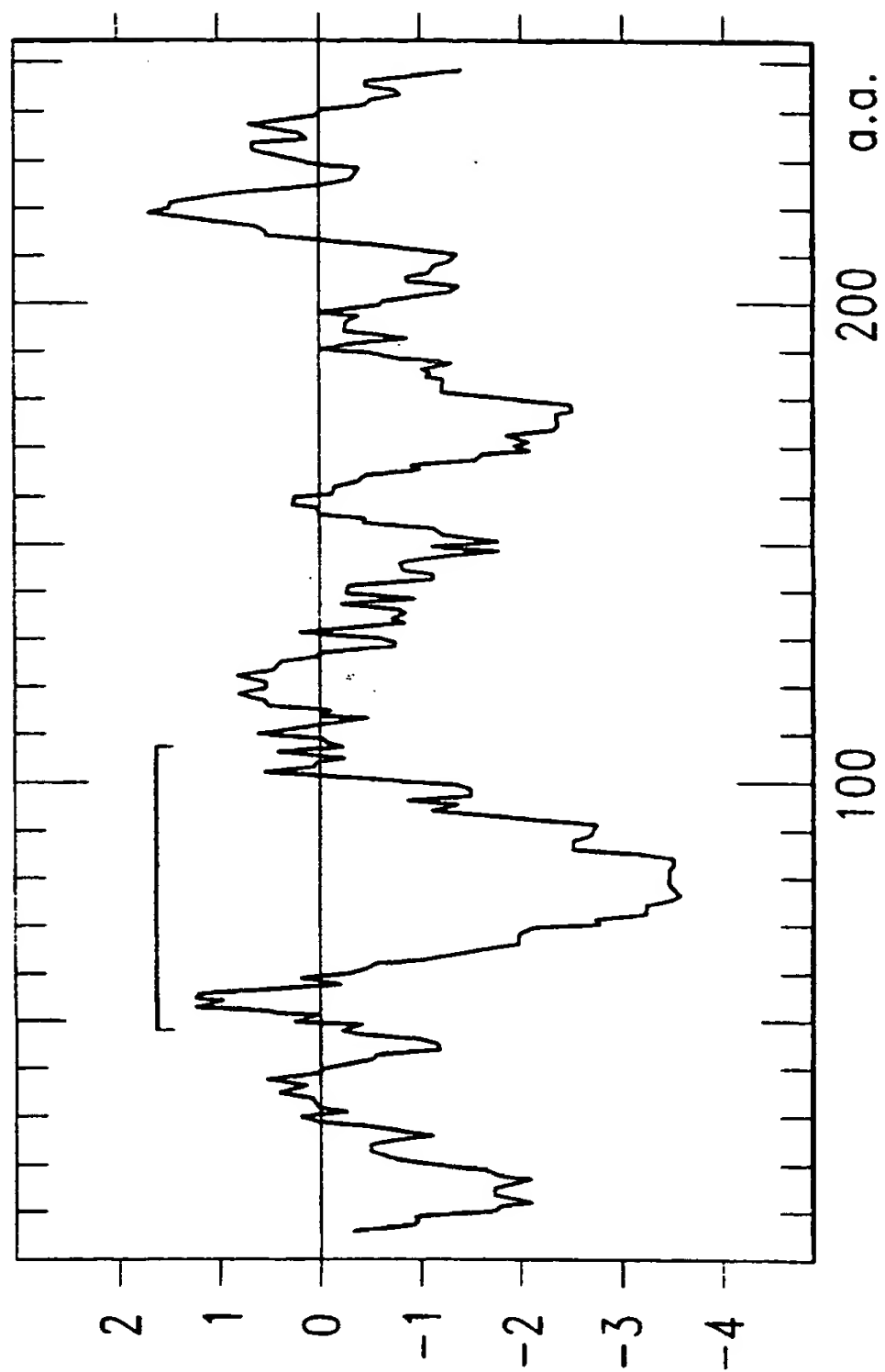


Fig. 33B



Hydrophobicity profiles of Capsides (Kyte - Doolittle)

Crrv

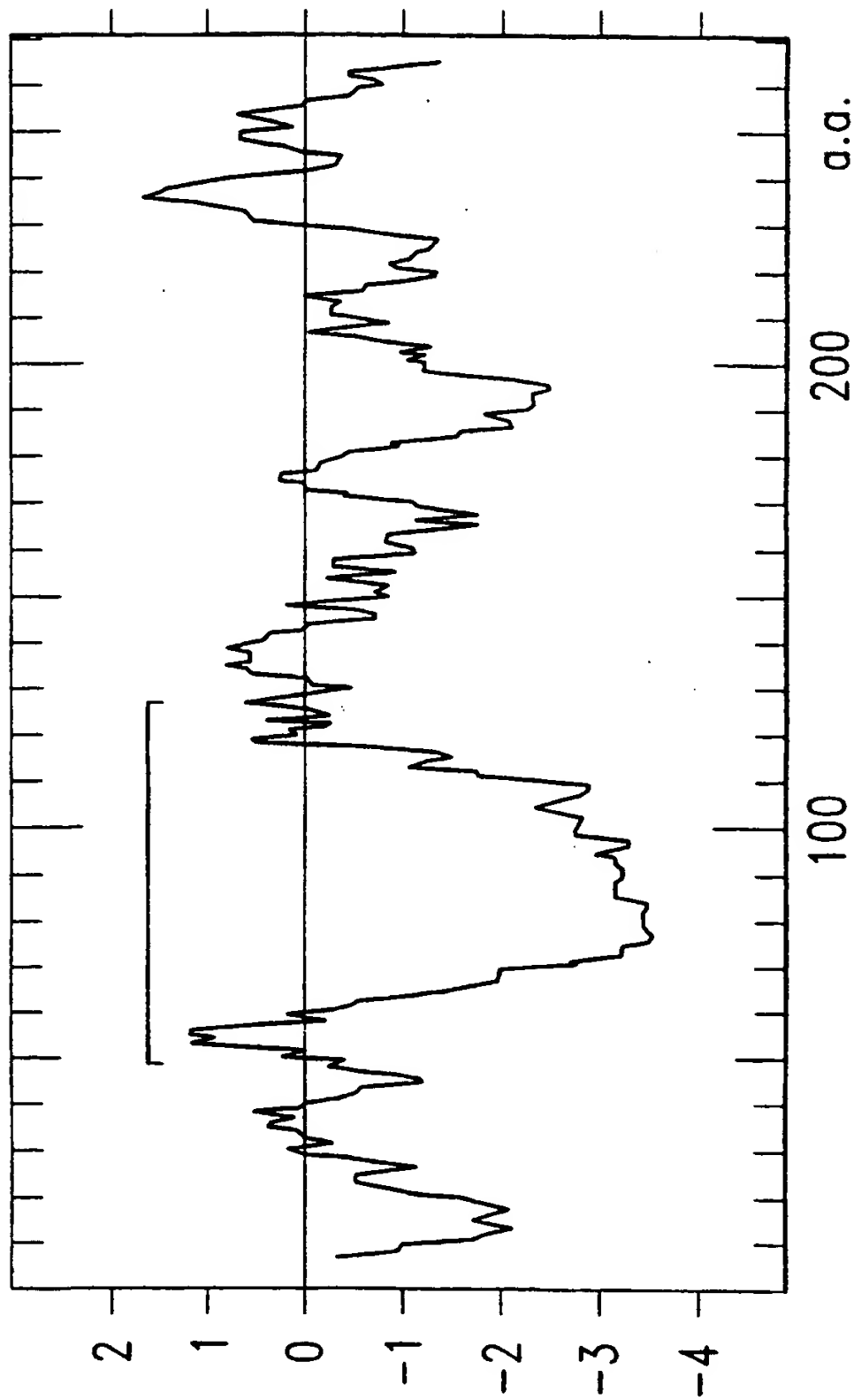
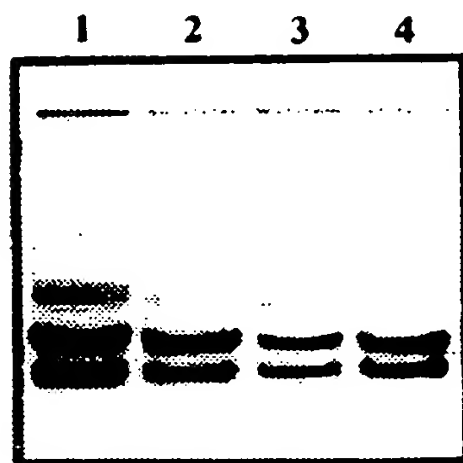


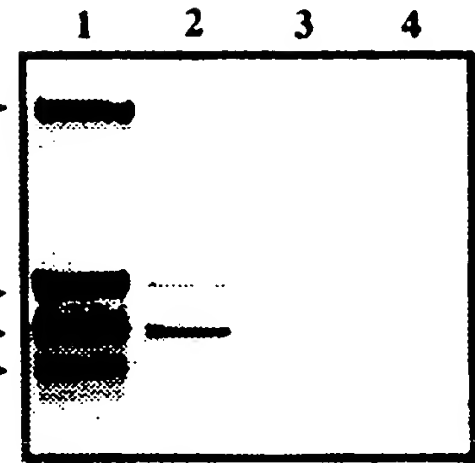
Fig. 33A

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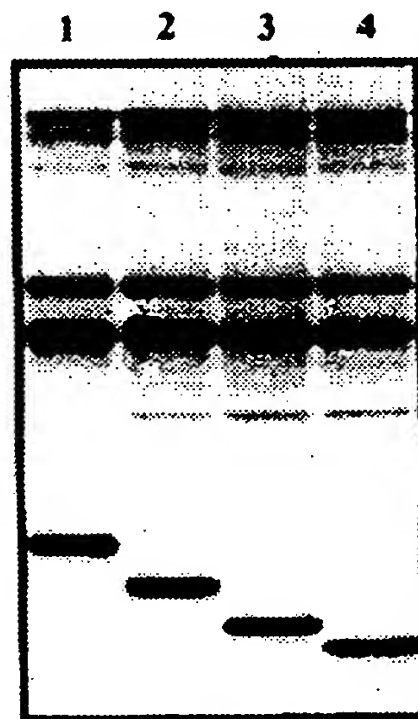
## RNAs in transfected cells



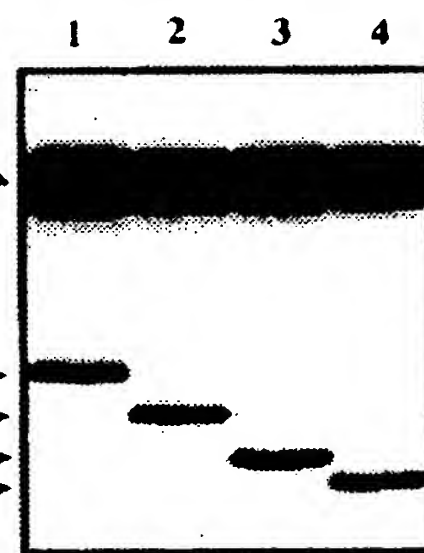
## RNAs in viral particles

*Fig. 31A**Fig. 31B*

## Proteins in transfected cells



## Proteins in viral particles

*Fig. 32A**Fig. 32B*

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# Inducible Intracellular Expression of Alphavirus Vector RNA (Two-Level Regulation)

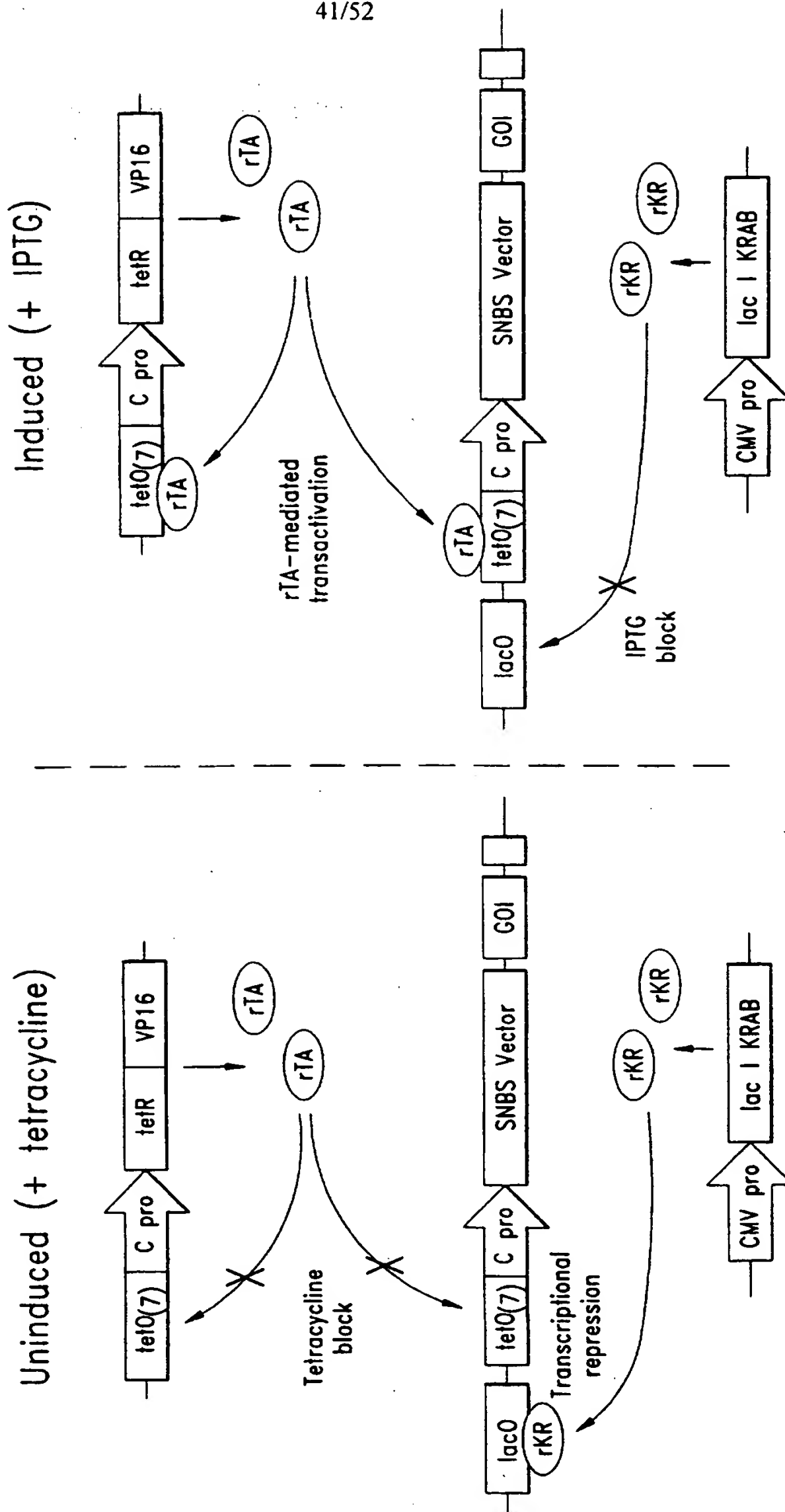


Fig. 30

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# Inducible Intracellular Expression of Alphavirus Vector RNA (Single-Level Regulation)

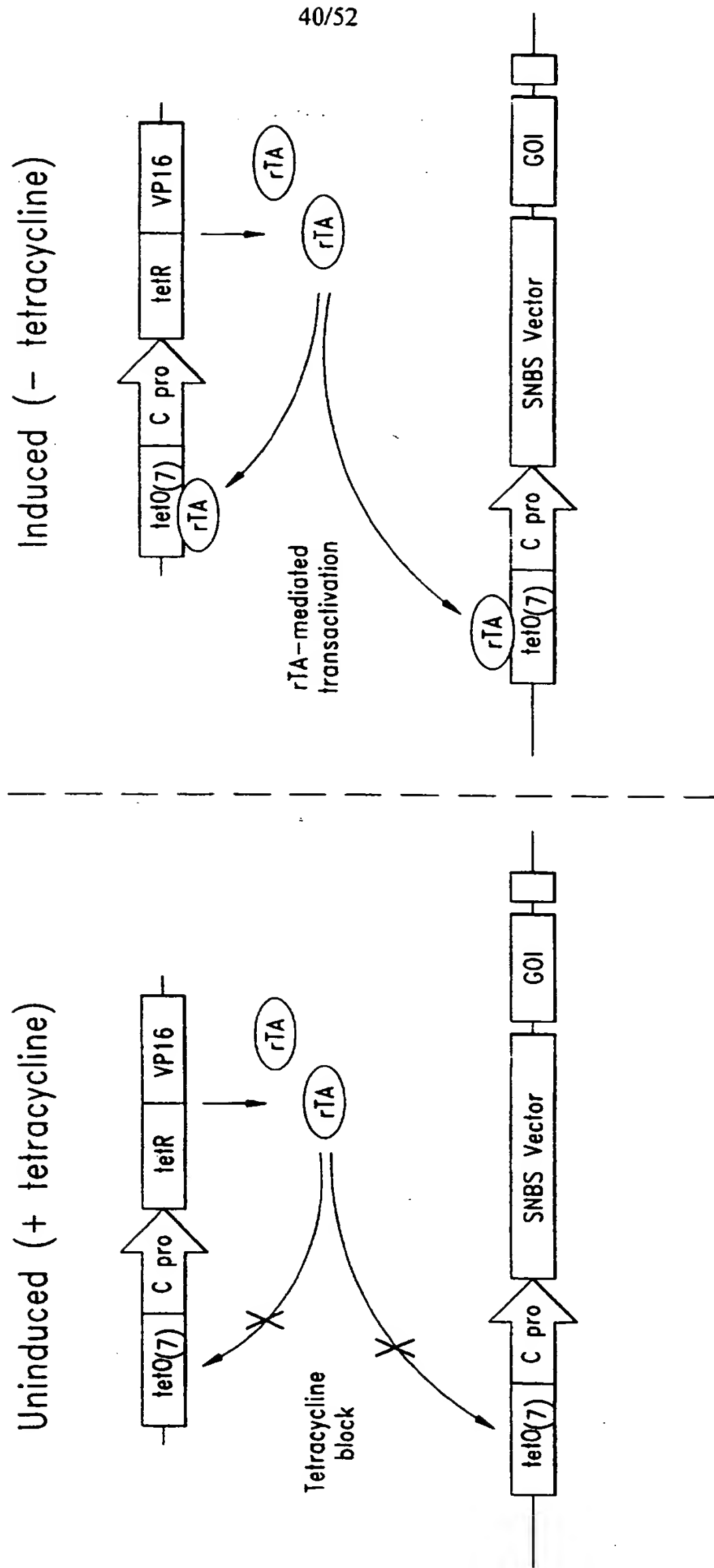
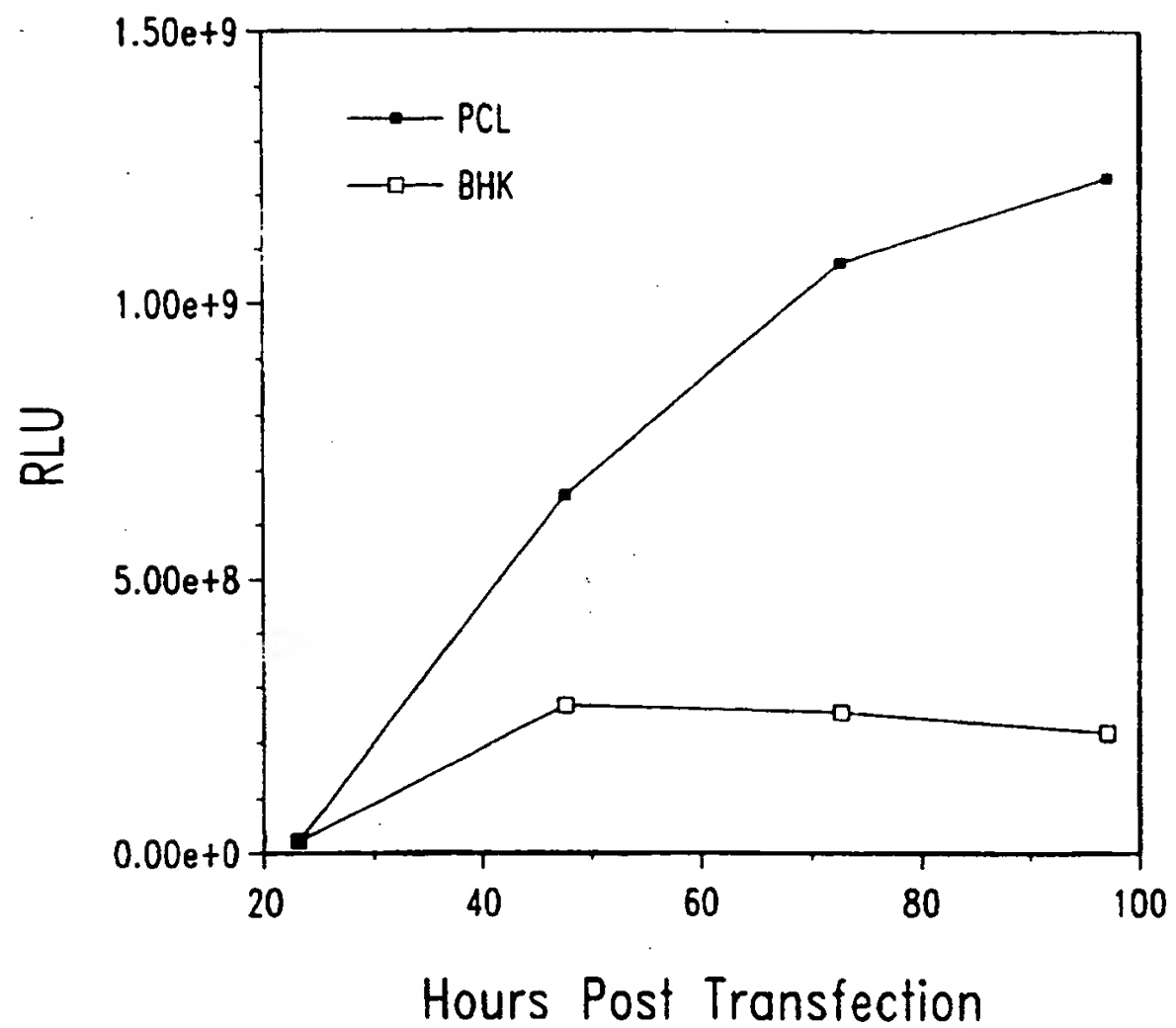


Fig. 29

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Amplification of Reporter Gene Expression  
by Transfection of PCLs with ELVS-lac Z

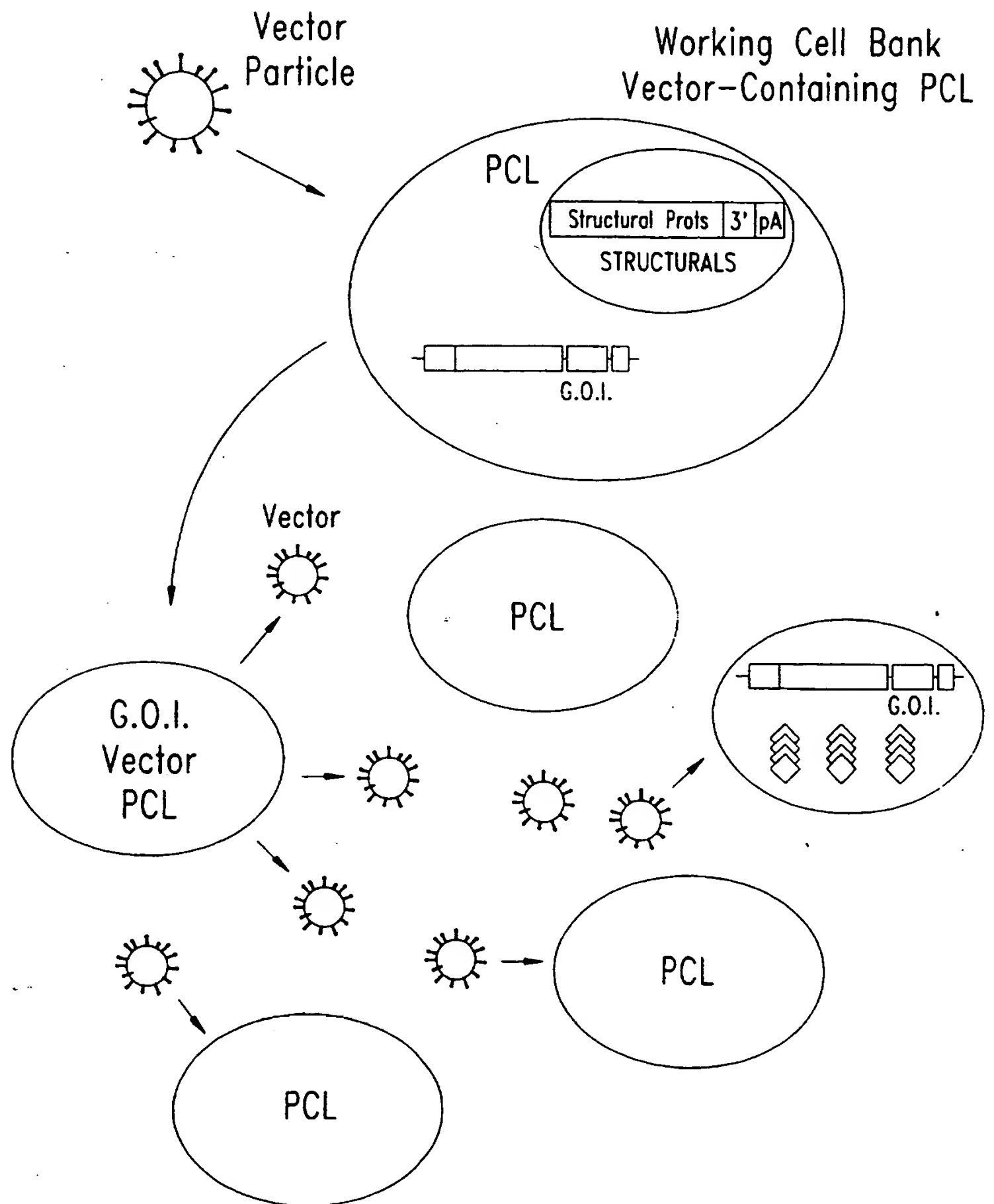


*Fig. 28*

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# Sequential Amplification and Recombinant Protein Expression Using SNBS™ Vectors and PCL



*Fig. 27*

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<u>Data-</u> <u>replicon (vector)</u>	<u>helper(all tRNA 5')</u>	<u>inf units/ml</u>	<u>pfu/ml</u>
1. SINrep/LacZ	DHBBCrrvSin-gps	5x10 <sup>-8</sup>	1.5x10 <sup>-6</sup> pinpoint
2. " "	DHBBCsin + DHBB(CdeletedRRVSin-gps)	5-10x10 <sup>-9</sup>	1.5x10 <sup>-7</sup> tiny
3. Sinrep/capsid	DHBB(CdeletedRRVSin-gps)		6x10 <sup>-7</sup> normal size

the latter (as well as gels) show that only the Sindbis virus capsid gets incorporated into extracellular particles when both are present in the transfected cell.

### III constructs

A Toto1101 - sites for the insertion of the RRV capsid gene

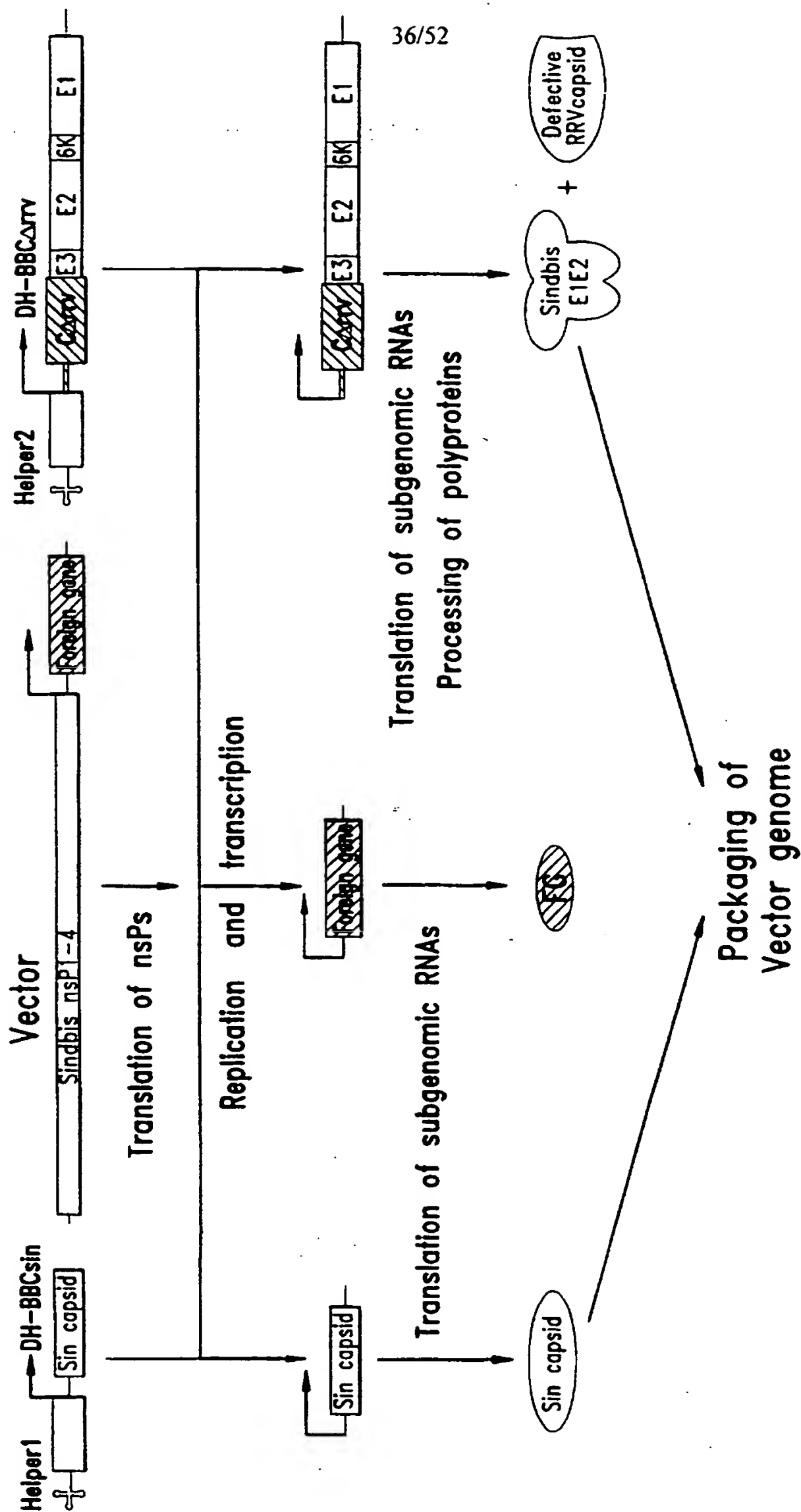
B RRV - sites used for cloning of the RRV capsid gene

C chimeras 1) complete RRV capsid gene  
2) RRV capsid gene nts 7835-7888 deleted.

*Fig. 26*

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# Electroporation



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Fig. 25